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TITLE: Feasibility Studies in Development of a Temporary  
Implantable Lactate Sensor Biochip for Monitoring  
During Hemorrhage

PRINCIPAL INVESTIGATOR: Anthony Guiseppi-Elie, Sc.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University  
Richmond, VA 23284-3031

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## INTRODUCTION

Following injury that results is tissue hypoxia, interstitial lactate levels increase and are the main source of metabolically-produced acid responsible for tissue acidosis. Lactate levels have also been found to correlate with the severity of injury, including hemorrhage. Small molecules such as lactate and glucose exists in equilibrium between the interstitial and vascular compartments when systemic levels are elevated. Interstitial levels of lactate should thus be reflective of systemic levels when whole body tissue hypoxia occurs such as in hemorrhage. The ability to monitor *in vivo* lactate in the interstitium is possible but current methods are either not clinically feasible for prolonged measurement or are unreliable because they suffer from deleterious effects of endogenous interferences and fouling. The goal of this research is to develop an implantable lactate-sensing biochip for temporary implantation and capable of telemetered reporting of local lactate levels. These biochips will be temporarily injected/implanted into a skeletal muscle bed such as the deltoid muscle of rats. Lactate levels will be continuously monitored for implantation periods varying from several hours to 3 months. This will include testing of the sensor in a model of severe hemorrhagic shock with comparison of sensor-recorded levels of lactate to systemic levels of lactate and whole body oxygen debt. A suitable MEMS antenna will be developed and demonstrated in parallel.

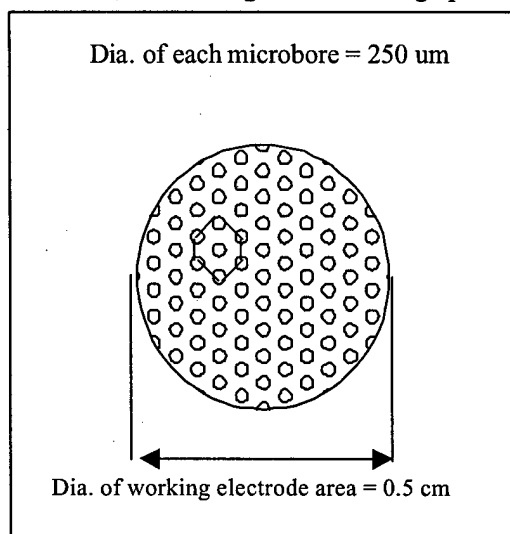
## BODY

**Task 1.** To integrate the "bio-smart" lactate-specific membranes with the microfabricated biochip, package the chip and perform pre-implantation *in vitro* testing and characterization

### a. Produce microlithographic "microbore" biochip substrates

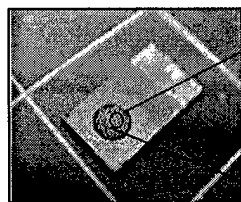
We have fabricated prototypes (macro-electrodes) of the microbore design of the working lactate biochip electrode. The microbore design consists of circular holes that are arranged in a hexagonal-close packed (hcp) array. Each microbore has a diameter of 250  $\mu\text{m}$ , with the spacing between holes of 500  $\mu\text{m}$  from center on center (Figure 1). These holes are contained within a defined working area of 0.19  $\text{cm}^2$ . Such an arrangement yields the maximum microbore hole packing density of 74% within the defined working area.

Planar metal electrodes (PMEs) were photolithographically processed with photoresist to create the conducting working electrode window while the rest of the electrode, excluding the bonding pad region, was insulated with resist. This was performed by coating the electrode with negative photoresist (NR7-1000PY, Futurrex), then spinning at 4000 rpm for 40 seconds under vacuum to establish a thin, even coating of photoresist. The electrode was then immediately soft baked at 115°C for 60 seconds on a hot plate. To generate the microbore pattern over the working electrode area, an optical photomask, designed in both SolidWorks and AutoCAD software and printed on an overhead transparency (Figure 1), was aligned over the resist-coated platinum electrode and exposed to UV light (366 nm, UVP crosslinker) for 3 minutes. Following UV treatment, the electrode was again soft baked (100°C, 60 s) and the negative photoresist-coated regions developed by immersing the electrode in water-based metal-ion-free developer solution (RD6 Developer, Futurrex Inc.,

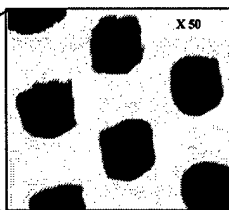


**Figure 1:** Design of microbore electrode mask using AutoCAD R-14 software. Microbore holes are arranged in a hexagonal close packed (hcp) configuration

Franklin, NJ) for 50 seconds with mild agitation. The developed electrodes were then rinsed thoroughly with deionized water and blown dry with nitrogen. The final treatment involves platinization of the microbore holes via controlled potential coulometry using a current density of 21  $\text{mA}/\text{cm}^2$  in a solution of hexachloroplatinic acid (YSI 3140 platinizing solution) to yield the microbore pattern shown in figures 2 & 3. A manuscript detailing the design, fabrication, and analytical performance of the final microbore biochip integrating working, counter and reference electrodes has been accepted for publication [1] (see appendix 1).



**Figure 2:** Photograph of microbore electrode after photolithographic processing and platinization

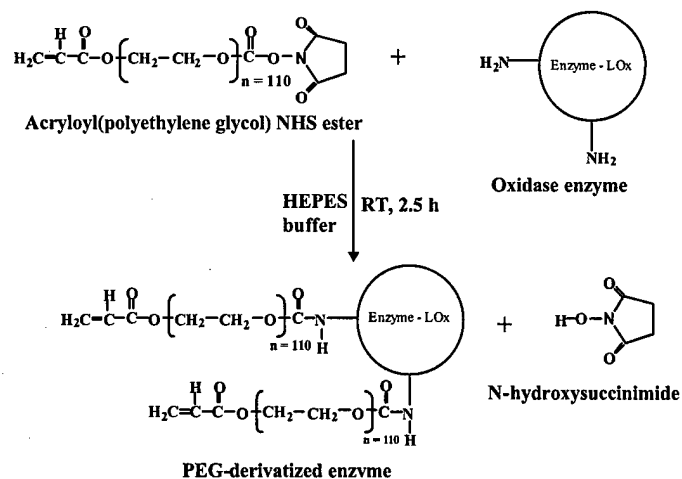


**Figure 3:** Blowup of photopatterned microbore "holes"

- b. **Prepare and cast 12 different formulations of composite bioactive lactate sensing membranes on the biochips and evaluate the *in vitro* analytical performance of each biosensor formulation, including biocompatibility testing of materials**

#### 1. Derivatization of lactate oxidase enzyme

Lactate specific (lactate oxidase - LOx) biosensor has been previously demonstrated with quantitative response to lactate in phosphate buffered saline. One major issue has been the relative lack of solubility of the lactate oxidase enzyme in the monomer mixture prior to casting on the electrode and subsequent polymerization. This has resulted in segregation (precipitation) of the enzyme leading to two phases in the gel membrane. A novel approach has been adopted that resolves this problem. First, derivatize the enzyme by covalently linking acryloyl-polyethylene glycol(PEG)-NHS ester to available lysine residues on lactate oxidase; typical molar ratio of 4:1 (PEG : enzyme). The NHS-ester part reacts with primary amines found on lysine (an amino acid) residues of the protein. Scheme 1 summarizes this enzyme PEGylation reaction. This has a two-fold effect: (i) the introduction of polyether functionalities that aid in solubility through extensive hydrogen bonding with the aqueous environment, and (ii) the

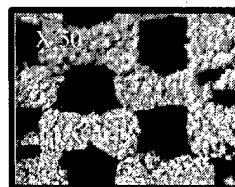


introduction of an acrylate moiety at the distal end that, in effect, "monomerizes" the enzyme, allowing it to be covalently incorporated into the hydrogel network during UV-induced network formation. This is the application of a novel technique of enzyme immobilization within hydrogels in which the enzyme is solubilized and also tethered within the gel.

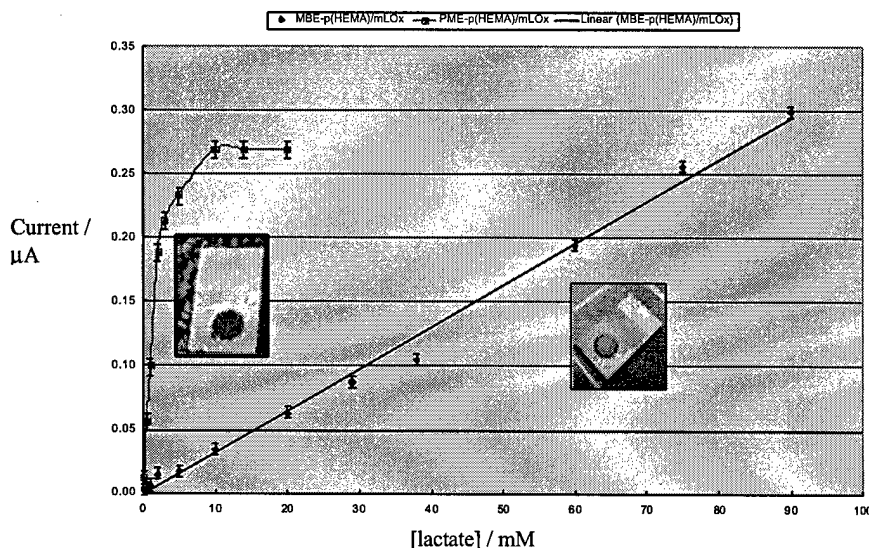
**Scheme 1:** PEGylation of lactate oxidase enzyme

## 2. Integration of derivatized enzyme as sensing membrane with microbore biochip – In vitro analytical characterization studies

The monomerized LOx (mLOx) was incorporated into the hydrogel formulation (96:3:1 mol% HEMA:TEGDA:PEGMA) as previously outlined and spin coated onto the active electrode surface of the microbore sensor to generate a lactate-specific sensing membrane upon subsequent polymerization (Figure 4). The response of the microbore biochip to varying lactate concentrations was evaluated in phosphate buffered KCl (0.01 M in each component, pH 7.2, 28°C). The microbore biosensor exhibited an extensive linear dynamic response range up to 90 mM lactate (Figure 5), with a sensitivity of 3 nA mM<sup>-1</sup> and an average response time ( $t_{95}$ ) of 50s. The detection limit was calculated to be  $8.0 \times 10^{-4}$  M. By comparison, the planar electrode design coated with the same mLOx-hydrogel membrane and operating under identical conditions showed linearity in amperometric response up to only 3 mM lactate, with a sensitivity of 66 nA mM<sup>-1</sup>. The planar sensor had a detection limit of  $9.0 \times 10^{-5}$  M ( $3S_{y/x}/\text{slope}$ ) and a response time ( $t_{95}$ ) of 75 s. The analytical performance characteristics of both biosensor designs are compared in Table 1.



**Figure 4:** Optical micrograph of microbore sensor active surface coated with mLOx-hydrogel membrane



**Figure 5.** Calibration curves for microbore lactate biosensor vs. planar lactate biosensor

**Table 1:** Comparison of analytical performance of the two biosensor devices

Sensor	Linear range (mM)	Sensitivity (nA mM <sup>-1</sup> )	Detection limit (mM)	Response time (s)	Regression equation
Planar	0.1 – 3.0	66.2	0.09 mM	75	$y = 0.0662x + 0.0208$
Microbore	1.0 – 90.0	3.3	0.8 mM	50	$y = 0.0033x - 0.0008$

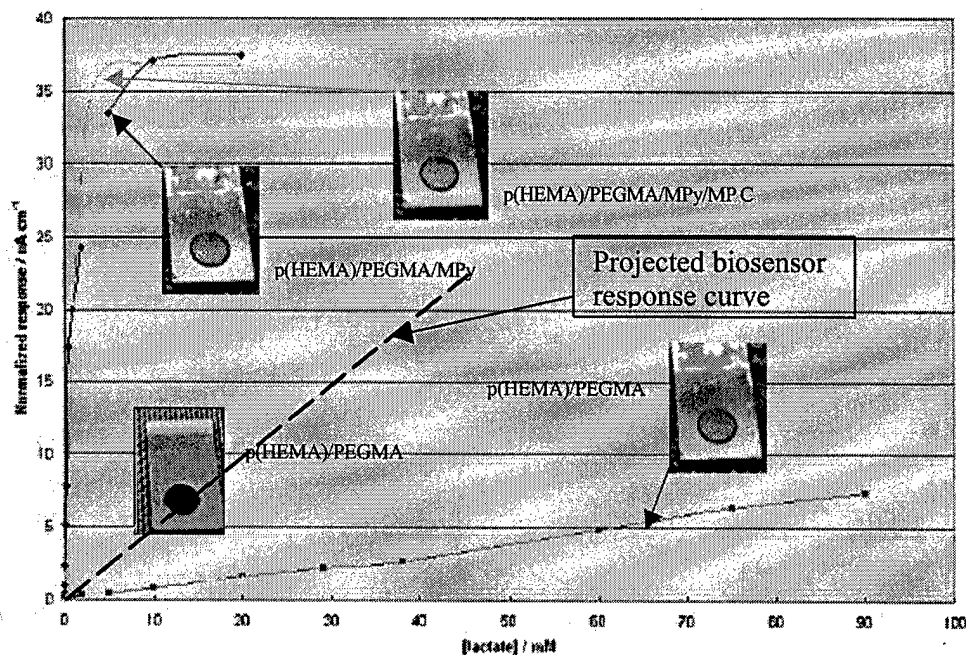
Thus by simply changing the electrode working geometry (and corresponding active sensor area) from a planar area to a microbore arrangement, we realize a dramatic 30-fold increase in the lactate biosensor's linear response range. Also significant is the reduction in sensitivity (22-fold decrease) of amperometric responses obtained with the microbore electrode.

We continued to evaluate the performance of the microbore lactate biosensor by modifying the hydrogel composition to include the biocompatilizing moiety:- 2-methacryloyloxyethyl phosphorylcholine (MPC), and the bifunctional monomer 2-methacryloyloxyethyl pyrrolylbutyrate (MPy). Briefly, the monomerized LOx (mLOx) was incorporated into the hydrogel formulation (94:3:1:1:1 mol% HEMA:TEGDA:PEGMA:MPy:MPC) and applied to the active electrode surface of the microbore sensor design, followed by UV-induced polymerization of the bioactive hydrogel sensing layer. The response of the resulting microbore biosensor to varying lactate concentrations was evaluated in phosphate buffered KCl (0.01 M in each component, pH 7.2, 28°C). The MPC-containing microbore biochip exhibited a linear response to lactate concentrations up to 3.0 mM lactate, with a response time ( $t_{95}$ ) of 65s and a detection limit of  $1.0 \times 10^{-5}$  M. Of interest is that with the subtle inclusion of the biocompatilizing moiety, MPC, into the composite hydrogel formulation, we observe a six-fold increase in the linear dynamic lactate response range compared to a microbore biosensor containing the conducting polymer component, MPy, but no incorporated MPC. However, the present biosensor exhibited a three-fold decrease in sensitivity ( $0.48 \mu\text{A mM}^{-1}$ ) compared to the biosensor containing MPy but with no MPC ( $1.412 \mu\text{A mM}^{-1}$ ). The following table (Table 2) compares the analytical performance characteristics of the four types functional biosensors fabricated to date. From this table, and in conjunction with the plot of the normalized response curves of the four biosensors (Figure 6), it can be concluded that the desired lactate biochip should be fabricated using a bio-smart hydrogel formulation that results in a response profile with sensitivity and linear dynamic range as projected.

**Table 2:** Comparison of analytical performance of the various biosensor devices.

Sensor	Membrane	Linear range / mM	Sensitivity $\mu\text{A mM}^{-1}$	Detection Limit / mM	Response Time / s	Regression Equation
PME	P(HEMA)-PEGMA	0.1 – 3.0	0.0662	0.0900	75	$y = 0.0662x + 0.0208$
MBE	P(HEMA)-PEGMA	1.0 – 90.0	0.0033	0.8000	50	$y = 0.0033x - 0.0008$
MBE	P(HEMA)-PEGMA-p(MPy)	0.002 – 0.5	1.4122	0.0018	70	$y = 1.4122x + 0.018$
MBE	P(HEMA)-PEGMA-p(MPy)-p(MPC)	0.02 – 3.0	0.4749	0.01	65	$y = 0.4749x + 0.0996$

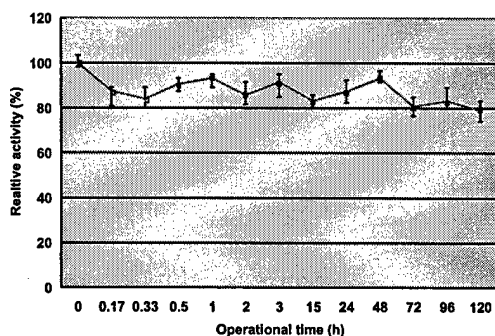




**Figure 6** Normalized response curves for the various lactate biochips with insert of idealized lactate response profile

### Operational and Storage Stability

The lactate microbore biosensor prepared from composite hydrogel composition 94:03:01:01:01 mol% HEMA:TEGDA:PEGMA:MPy:MPC was evaluated for its continuous operational response and stability in a simple, simulated physiologic environment. The biosensor was placed into a three-cell electrochemical setup containing phosphate buffered saline solution, (0.1 M  $\text{NaH}_2\text{PO}_4$  + 0.15 M NaCl) at pH 7.3 and maintained at 37°C with continuous stirring (200 rpm). The response of the biosensor to injected 10 mM lactate concentrations was recorded at regular intervals over a five (5) day period of continuous operation (polarized at +700 mV vs. Ag/AgCl, 3M Cl). Figure 7 shows the observed operational response of the biochip.

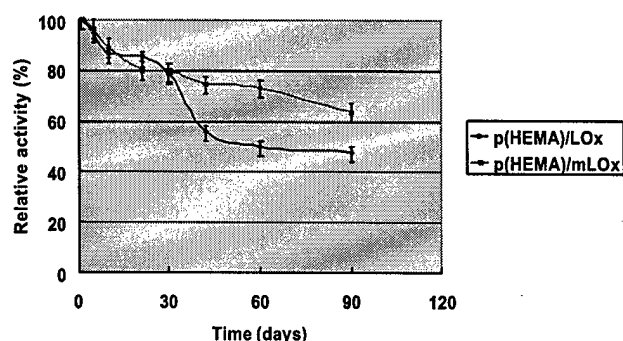


**Figure 7:** Operational stability of the lactate biosensor in phosphate buffered saline, pH 7.3, 37°C. Relative activities are expressed as the ratio of the amperometric response to 10 mM lactate divided by the initial amperometric response to 10 mM lactate (at  $t = 0$  h).

After 5 days (120 h) of continuous incubation in the PBS environment at 37°C, the biosensor still retained greater than 80% initial activity, with consistent response times ( $t_{95}$ ) of ca. 60 s.

The ability of the derivatized LOx (mLOx) to retain enzymatic activity within the micro-environment of the hydrogel membrane was also evaluated over a storage period of three (3) months. This was done by measuring the amperometric current generated by the biosensor in response to a 3 mM standard lactate solution over this time period. As shown in Figure 8, the derivatized enzyme (p(HEMA)/mLOx) retained greater than 60% of its initial activity after three months of storage in phosphate buffered saline at 4°C. For comparison the biosensor containing underivatized LOx (p(HEMA)/LOx) retained less than 50% initial activity for the same length of storage time under identical conditions.

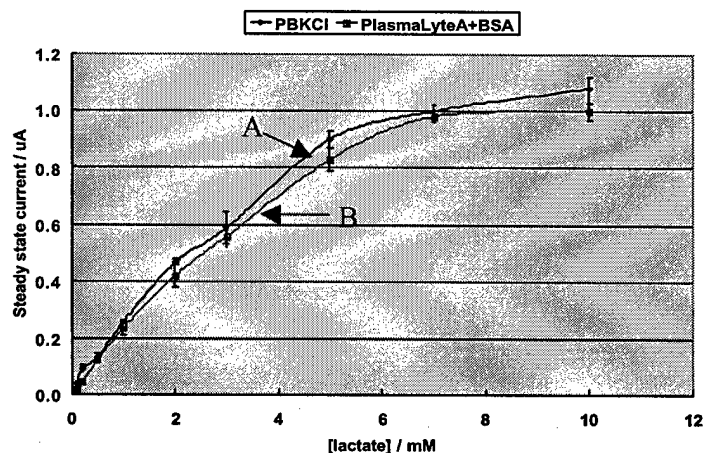
**Figure 8:** Comparison of storage stability profiles for biosensors containing monomerized LOx (mLOx) and underivatized LOx



#### Characterization of microbore biochip sensor in simulated physiologic medium

We investigated the effect of serum protein on the biochip performance. A freshly fabricated lactate biosensor was prepared from composite hydrogel composition 94:03:01:01:01 mol% HEMA:TEGDA:PEGMA:MPy:MPC. The amperometric response of the biosensor to various lactate standards was tested first in 0.1M phosphate buffered KCl, pH 7.4 at 37°C, then in Baxter Healthcare *Plasma-Lyte® A* Interstitial Fluid, pH 7.4 at 37°C containing 10g/L bovine serum albumin (BSA). Figure 9 shows the respective response curves.

**Figure 9:** Response curve for lactate biosensor measured in (A) PBKCl, pH 7.4 at 37°C and (B) *Plasma-Lyte® A* Interstitial Fluid, pH 7.4 at 37°C containing 10g/L BSA.



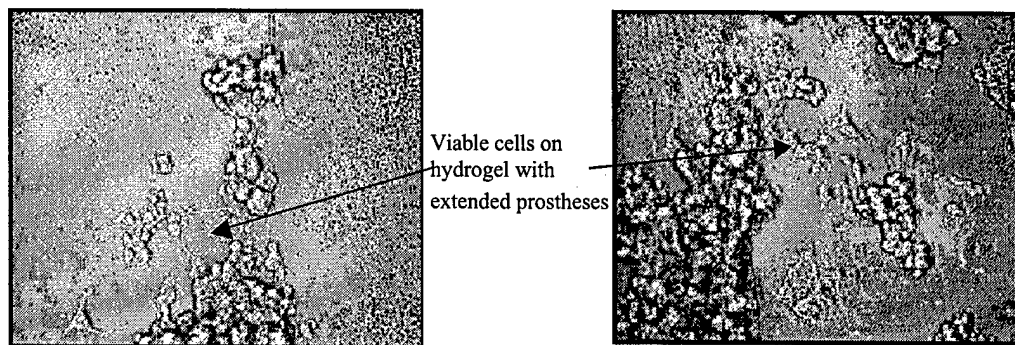
In PBKCl, the biosensor displayed a linear dynamic response range up to 3.0mM lactate ( $r^2 = 0.984$ ) with a sensitivity of 0.20  $\mu\text{A}/\text{mM}$  lactate and a response time ( $t_{95}$ ) of 70s. The same biosensor tested in *Plasma-Lyte*<sup>®</sup>A fluid containing 10g/L BSA showed a response profile that was not significantly different from that in buffer. The linear dynamic response range up to 3.0mM was preserved and there was no observed increase in response time. Of particular interest is the retention of 95% of the sensitivity (0.19  $\mu\text{A}/\text{mM}$  lactate) attained with the biochip functioning in buffer medium.

### 3. Biocompatibility testing of multifunctional "bio-smart" materials

#### Cell viability studies

To evaluate the biocompatibility of the polymer composites, a suitable chamber or culture plate has to be prepared to accommodate the proposed formulations. We selected commercially available 8-well cell culture plates to culture either Rat Pheochromocytoma (ATCC Designation: PC-12; ATCC Number CRL-1721) or Human Muscle Fibroblast cells (ATCC Designation: SJCRH30 [RC 13, RMS 13, SJRH30] ATCC Number CRL-2061). Two commercial 8-well culture plates were chemically derivatized and subsequently coated with thirteen (13) different polymer formulations of the biochip biomaterials. The biomaterials all possessed a similar polymeric backbone consisting of cross-linked poly(2-hydroxyethyl methacrylate) containing varying mole fractions of the biocompatilizing moieties; 2-methacryloyloxyethyl phosphorylcholine-containing monomer (MPC) and polyethylene glycol methacrylate (PEGMA). The capability of the derivatized culture plates to support the growth and viability of two types of cells, human epithelial cells and human muscle fibroblast cells were tested.

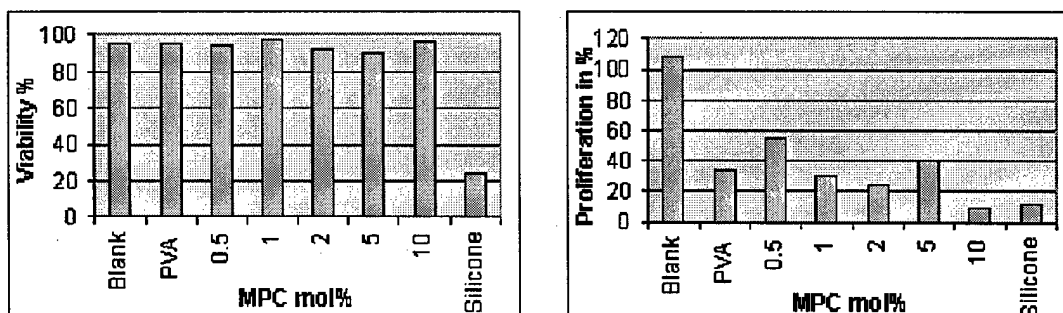
As shown in Figure 10, epithelial cells grew and proliferated in wells that were coated with hydrogels containing **2-methacryloyloxyethyl-phosphorylcholine (MPC) monomer**.



**Figure 10:** Optical micrograph of culture well coated with MPC-containing hydrogel showing growth and proliferation of human epithelial cells.

Human muscle fibroblast cells (type CRL-2061) (400  $\mu\text{L}$  culture medium volume) were introduced into each well of an 8-well cell culture plate coated with hydrogels having varying mol% of MPC (initial cell seeding density was  $5.0 \times 10^5$  cells/ml). The cells were

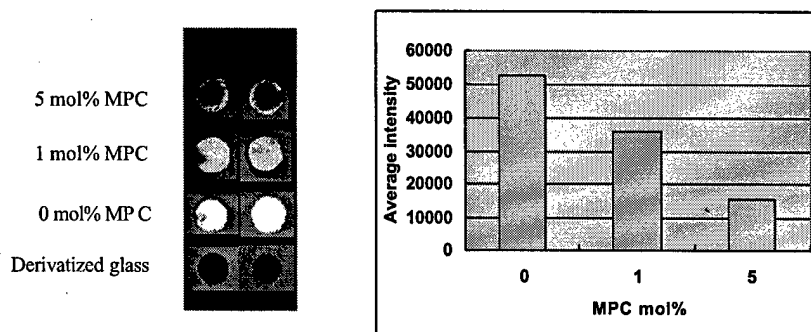
inspected after 48h and 72h and the resulting cell viability and proliferation results are graphed in Figure 11.



**Figure 11:** Viability and proliferation results for human muscle fibroblast cells cultured on derivatized surfaces coated with hydrogels of varying MPC content. PVA and silicone are used as - and + controls with underivatized glass (blank) as the reference surface.

### *Protein adsorption studies*

We evaluated the adsorption of three prominent extracellular matrix proteins; fibronectin, laminin and collagen, onto the composite hydrogels. In all cases, there was a progressive decrease of adsorbed protein onto the hydrogel coated surface as the mol% of MPC was increased as illustrated in Figure 20.



**Figure 20:** Fluorescence intensity profiles of hydrogel membranes (5mm dia.) containing different MPC concentrations after immersion in a 0.01 ug/mL solution of laminin for 1 hour.

### *In vivo implantation*

#### **Objective of this area of study:**

The objective of this area of study was to analyze the *in vivo* reactivity of the multifunctional "bio-smart" hydrogel materials that may be suitable candidates for the *in vivo* biochip.

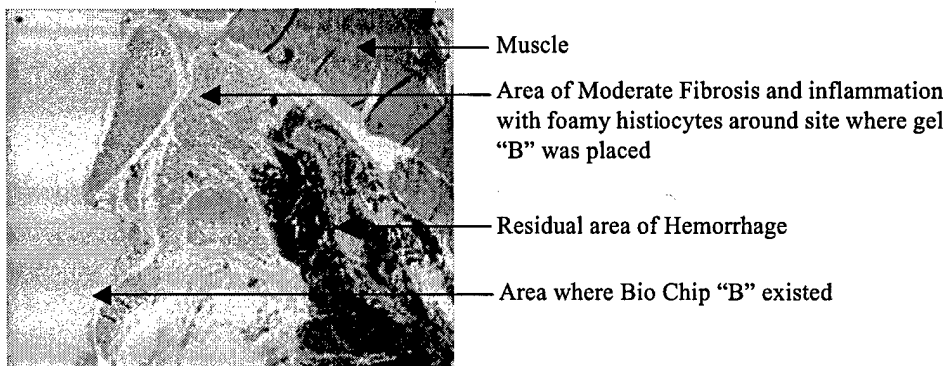
#### **Research Strategy:**

Hydrogel materials of various compositions were implanted in the medial trapezius muscle in rats as described by the approved VCU IACUC protocol. Briefly, all gels were rinsed 3 times in sterile saline and implanted in the anesthetized animal using sterile techniques. In the 1<sup>st</sup> phase of these studies, the animals were observed for 2 weeks and then the gel and surrounding tissues were excised and fixed in buffered formalin for histology. Any animal displaying overt signs of inflammation or discomfort (ie. redness, sensitivity to touch, heat or swelling in the area of implantation or partial or complete wound dehiscence) were sacrificed immediately and samples were harvested. It was decided that 2 implantable materials would be used for comparison to the test hydrogel samples. Poly vinyl alcohol (PVA) was chosen because it is a well known biomaterial that elicits a vigorous foreign body response with significant encapsulation and cellular penetration. Medical grade Silicone<sup>®</sup> was chosen to represent the opposite end of the spectrum because it is approved for human implantation and is known to produce a very minimal foreign body reaction. These 2 biomaterials were used as positive and negative controls with the full understanding that they are far removed from any consideration as potential platforms for the chemistry needed for the *in vivo* biosensor. The gross and histopathology findings were compared to the findings displayed by PVA and Silicone<sup>®</sup>.

#### **Results:**

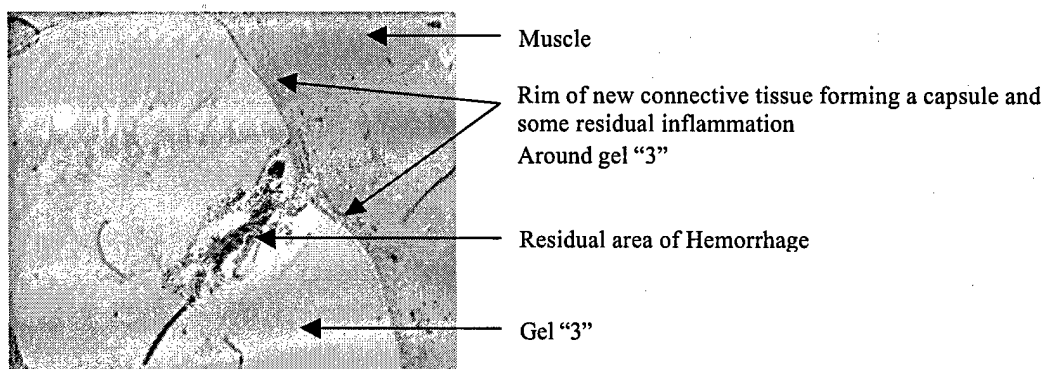
As shown in the following sample photomicrographs, all of the hydrogel materials elicited some degree of foreign body responsiveness. Materials B, 6 and 7, corresponding to compositions 97:03 mol% H:T, 97:03:0.01 mol% H:T:PMB, and 92:03:05:0.05 mol% H:T:MPC:PMB, caused a greater response characterized by a thick area of encapsulation. The "foamy histiocytes" seen around sample B suggests the presence of fat or lipid-like material in the area of implantation and could be secondary to cell necrosis. In contrast, samples 3 and 5 (96:03:01 mol% H:T:MPC and 97:03:0.01 mol% H:T:PMB) caused a lower response resulting in a thin band of encapsulation. It is important to note that none of the materials exhibited a "granulomatous" type of response that is characteristic of a very vigorous foreign body response. It is also important to note that the newly deposited connective tissue did not penetrate into the body of any of the test materials, but remained on the exterior.

It is remarkable that the detailed histologic findings agree closely with the gross findings that were recorded upon hydrogel explantation. Materials are now undergoing further testing where they will be implanted for 1 month. The critical question that needs to be addressed regards to what degree, if any, this encapsulation tissue may interfere with the ability of the detection platform to quantify tissue lactate and what increase in the time of the response will it cause. Additional studies are currently in progress.

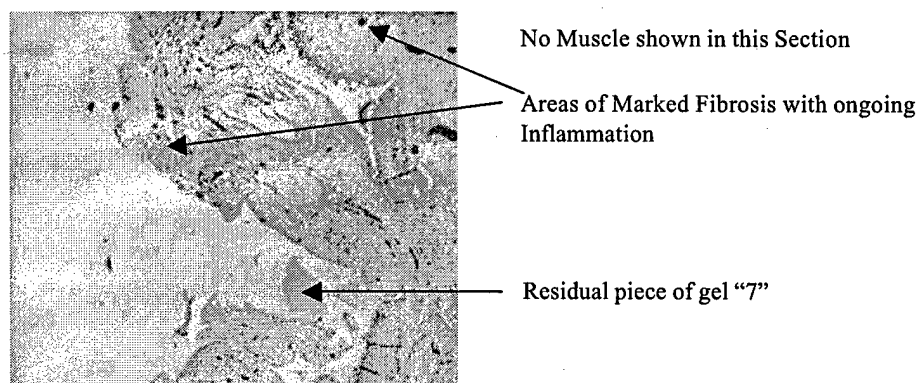


Hydrogel sample "B" – 97:03  
mol% HEMA:TEGDA

Path report stated "Slide number B is different and shows a collection of foamy histiocytes in addition to mild fibrosis and inflammation."



Hydrogel sample "3" – 96:03:01  
mol% HEMA:TEGDA:MPC



Hydrogel sample "7" – 96.5:03:0.5:0.05  
mol% HEMA:TEGDA:MPC:PMB

**Task 2.** To implant the lactate biochip intramuscularly into animal models and evaluate *in vivo* biosensor performance

**a. Surgically implant the packaged biochip device intramuscularly in adult Sprague Dawley rats and catheterize the animals for blood lactate sampling and infusion.**

A critical aspect of both Task #1 and Task #2.a. is the development of an animal model containing a chronic catheter implant allowing for infusion of glucose, lactate, and fluids and withdrawal of blood for sampling of the same over prolonged periods of time (up to one year). No such animal model or catheter implantation technique has been reported in the literature. Development of this animal model is critical to allow for long-term functional testing of the implantable biochip for glucose and lactate monitoring. The functional testing of the chip in this regard is to take place by challenging the animal's systemic circulation with glucose and lactate on a daily to weekly basis. This would allow for testing of the biosensor's response time, sensitivity and accuracy of measurement in the tissue interstitium against repeated measures of whole blood analysis of these same small analytes. Thus biosensor response degradation could be examined. The only viable means to do these repeated measures was to develop an animal model which would allow easy access to the animal's vasculature in a manner that would not require repeated surgeries, anesthetics, and stress.

The technique chosen and perfected over the last 12 months involves cannulation of the animal's inferior vena cava with a catheter which is then connected to a vascular access port buried subcutaneously in the nape of the animal's neck. The inferior vena cava is chosen because high blood flows passing over the catheter are required to prevent formation of blood clots over its tip. The model is a major surgical endeavor and requires meticulous surgical technique to prevent the possibility of infection and abdominal organ injury. The technique is presented in the pictorial below.

After considerable effort, we have perfected this technique and have determined the best catheters and vascular port materials for use. As of this report, we are monitoring 12 animals with the chronic catheter implants with implant lifetimes ranging from 4 weeks to 3 months. Catheter patency is tested daily for the first three weeks and then biweekly after that. To date only one catheter implant as failed patency testing. This represent a greater than 90% success rate. All animals have tolerated the implantation and gain weight similar to animals matched for age without implants. Despite frequent manipulation of the vascular access port, no evidence of local or systemic infection have been noted. The use of these catheter implants will also be valuable when the acute hemorrhage experiments are executed for the measurement and comparison of oxygen consumption with interstitial and systemic lactate levels.

A manuscript describing this technique and its utility is currently being written and will be submitted once all animals have passed the three months implantation period.

**Selected steps of catheter implantation into the inferior vena cava  
(using maintenance free VAP + CBAS tubing)**



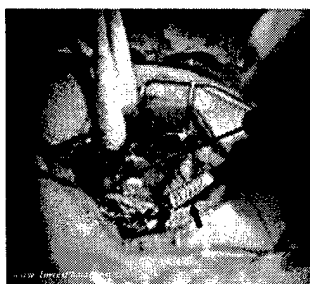
1

Vascular access port (VAP) connected to a CBAS tubing (catheter) after routing.



2

A straight and sharp tweezer passing through the abdominal wall to pick the catheter end, which will be inserted into the Vena Cava.



3

Left and right VC branches are clamped  
The forceps grasps the vessels and the 23G needle punctures the vena cava wall.  
Q tips swabs the blood.



4

Catheter tied on the psoas muscle, gauze pads removed and intestines are back to the original/physiological position. VAP with needle are showed.



## KEY RESEARCH ACCOMPLISHMENTS

- Enhancement of robustness and stability of lactate oxidase enzyme through PEGylation and hydrogel immobilization.
- Fabrication of amperometric lactate biochip sensors that possess superior sensitivity to other lactate biosensors reported in the literature.
- Design and fabrication of microbore biochip devices that offer increased linear dynamic response range towards analytes compared to the planar electrode design
- Design and synthesis of multifunctional “bio-smart” hydrogel materials that enhance biocompatibility through negligible protein adsorption.
- Development of novel catheter implantation surgical procedures into inferior vena cava of anesthetized animal model, allowing for long-term functional testing of the implantable biochip for glucose and lactate monitoring.
- The glucose biochip exhibited a linear dynamic range of 0.10 - 13.0 mM glucose with a response time of 50 s. The immobilized glucose oxidase within the bio-smart hydrogel yielded a Michaelis-Menten constant,  $K_{m(app)}$ , of 35 mM, suggesting that the immobilized glucose oxidase enzyme exhibits catalytic activity not significantly different from that for the native, solution-borne enzyme (33 mM).

## REPORTABLE OUTCOMES

### *Manuscripts*

Guisseppi-Elie, A.; Brahim, S.; Slaughter, G.; Ward, K. R. "Design of a subcutaneous implantable biochip for monitoring of glucose and lactate" (2004) *IEEE Sensors Journal* (accepted for publication).

### *Presentations*

#### **"Feasibility Studies in Development of an Implantable Lactate Biochip Sensor for Monitoring During Hemorrhage"**

Anthony Guisseppi-Elie<sup>1,2</sup>, Sean Brahim<sup>2</sup>, Sheena Abraham<sup>2</sup>, Gymama Slaughter<sup>2</sup>, Kevin Ward<sup>3</sup>, R. Wayne Barbee<sup>3</sup>, Robert Diegelmann<sup>4</sup>, Felix Miranda<sup>5</sup>, Rainee Simons<sup>5</sup>

<sup>1</sup>Department of Chemical Engineering and <sup>2</sup>Center for Bioelectronics, Biosensors and Biochips, Virginia Commonwealth University, Richmond, Virginia 23284-3038,

<sup>3</sup>Department of Emergency Medicine, Virginia Commonwealth University Reanimation Engineering Shock Center, Richmond, Virginia 23298, <sup>4</sup>Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia 232, <sup>5</sup>Applied RF Technology Branch, NASA Glenn Research Center, Cleveland, Ohio 44135

Presented at: ATACCC 2003 Conference, August 17-22, 2003, Tradewinds Island Grand Resort, St. Pete Beach, Florida.

### *Degrees obtained that are supported by award*

M.S. (Engineering) – Ms. Gymama Slaughter

Title: MODIFICATION OF GOLD ELECTRODE SURFACES FOR IMPROVEMENT OF NEURON-TO-ELECTRODE SIGNAL TRANSMISSION (NEST) – A. Guisseppi-Elie, Advisor

M.S. (Engineering) – Ms. Sheena Abraham (in progress)

Title: "Molecularly Engineered Hydrogels for Biocompatibility" - A. Guisseppi-Elie, Advisor

Ph.D. (Engineering) – Ms. Gymama Slaughter (in progress)

Title: "Impedimetric Detection of Neuron-to Electrode Signal Transmission" - A. Guisseppi-Elie, Advisor

### *Funding applied for based on work supported by award*

NSF 04-522 Sensors and Sensor Networks (\$375,000): Molecularly Engineered  
Hydrogels for Implant Biocompatibility  
Submitted: 02-26-2004. *A. Guiseppi-Elie, Principal Investigator*

This proposal is concerned with the molecular engineering of polymeric materials to convey multi-functionality in the design and application of the biorecognition layers of implantable biosensors. Specifically, it is concerned with the molecular manipulation of chemical composition to achieve the engineering property of biocompatibility in the sensing layer of implantable biosensors. This is an extremely challenging issue. The biorecognition layers of implantable biosensors must comprise bioactive molecules, in this case enzymes, that must remain stable and be bioactive for the duration of the implant. Moreover, they must be implanted and NOT elicit a significant foreign body response that will lead to capsule formation and loss of vascularization in the vicinity of the functioning device. This latter point leads to poor mass transport of metabolites (analytes of interest) to the functioning device and has thus far prevented the scientific community from achieving success with long-duration implantable glucose and /or lactate biosensors for closed-loop delivery of insulin and for physiologic status monitoring. We propose to molecularly manipulate poly(2-hydroxyethyl methacrylate) [p(2-HEMA)], a well-known non-cytotoxic polymer with familiar use in contact lens applications, to serve as the transducer-active membrane layer of implantable biosensors.

## CONCLUSIONS

We describe the design and fabrication of a microfabricated biochip that integrates soft-condensed, bio-smart hydrogels with solid-state microfabricated electrodes that has the potential for the continuous, long-term, *in vivo* monitoring of clinically important analytes such as glucose and lactate. The sensing membrane layer consists of, among other things, three main components; cross-linked p(HEMA) which serves as the hydrophilic network backbone, PEG chains that are tethered to the polymer backbone to stabilize enzyme activity and an MPC component that confers improved tissue biocompatibility. The enhanced biocompatibility of the sensing membrane afforded by the inclusion of the latter two components was demonstrated by 1) significant reduction in adsorption of extracellular matrix proteins; and 2) the apparent non-influence of BSA on the (continuous) analytical sensing performance of the biochip. The microbore electrode design, when applied to a lactate biosensor, demonstrated linearity up to 90 mM lactate, thirty-fold greater than the linear dynamic range for a corresponding planar lactate biosensor. The effect of enzyme PEGylation was apparent in the relative ease of solubilization into the monomeric formulation prior to biorecognition membrane fabrication. Physical entrapment of the PEGylated enzyme into the bio-smart hydrogel to produce the lactate biosensor resulted in enhanced operational and storage stabilities of the PEGylated enzyme lactate biosensor compared to the underivatized enzyme biosensor.

## REFERENCES

- [1] A. Guiseppi-Elie, S. Brahim, G. Slaughter, and K.R. Ward, "Design of a Subcutaneous Implantable Biochip for Monitoring of Glucose and Lactate," *IEEE Sensors Journal* **2004** accepted for publication.

## APPENDIX I -- A one page SUMMARY of this report

Lactate-specific microbore biochips have been fabricated that have been demonstrated to sense lactate over the dynamic range 1 mM to as high as 90 mM, with sensitivities typical of or greater than that of reported amperometric lactate biosensors ( $4 \text{ nA mM}^{-1}$  –  $1.4 \text{ } \mu\text{A mM}^{-1}$ ) and response time around 60 seconds. The sensing membrane of the biochips was tailored to resist protein adsorption and subsequent biofouling, while preserving biomolecule activity and stability. The microbore biochip was capable of detecting lactate in a simulated physiologic medium (Baxter Healthcare *Plasma-Lyte*<sup>®</sup>A Interstitial Fluid) containing up to  $10 \text{ gL}^{-1}$  bovine serum albumin, without any compromise to linear dynamic range, sensitivity, response time and operational stability for up to 3 hours. The immobilized enzyme, PEGylated lactate oxidase, retained greater than 65% initial activity after three months of storage. Subtle changes to the incorporated content of the biocompatibilizing moiety, MPC monomer, into the bio-smart hydrogel matrix resulted in significant variation to the amount of extracellular matrix protein adsorbed onto the hydrogel surface. Increasing the incorporated MPC monomer content from 0 mol% to 5 mol%, for example, yielded greater than 65% reduction of adsorbed laminin ( $0.01 \text{ } \mu\text{g mL}^{-1}$  stock solution). The biocompatibilizing character imparted by the inclusion of MPC monomer into the hydrogel membranes was also observed with the cell viability studies using human muscle fibroblast cells and human epithelial cells. Hydrogel formulations that included MPC monomer were found to support cell viability with only moderate proliferation. Intramuscular *in vivo* implantation studies of the hydrogel membranes in rat models showed only moderate inflammation and foreign body response of the explanted surrounding tissue after 2 weeks of implantation.

A novel catheterization and surgical technique has been pioneered that allows for long-term functional testing of the implantable biochip for dual glucose and lactate monitoring in the tissue interstitium of conscious rat models. The surgical technique involves cannulation of the animal's inferior vena cava with a catheter, which is then connected to a vascular access port buried subcutaneously in the nape of the animal's neck. To date, catheter patency with greater than 90% success rate has been achieved. The use of these catheter implants will also be valuable when the acute hemorrhage experiments are executed for the measurement and comparison of oxygen consumption with interstitial and systemic lactate levels.

**Design of a Subcutaneous Implantable Biochip for Monitoring of Glucose and Lactate**

Anthony Guiseppi-Elie<sup>‡,†,\*</sup>, Sean Brahim, Gymama Slaughter, and Kevin R. Ward<sup>†</sup>

Center for Bioelectronics, Biosensors and Biochips (C3B), <sup>‡</sup>Department of Chemical Engineering, and

<sup>†</sup>Department of Emergency Medicine, Virginia Commonwealth University, P.O. Box 843038, 601 West

Main Street, Richmond, Virginia 23284-3038

**ABSTRACT**

The design, fabrication and *in-vitro* evaluation of an amperometric biochip that is designed for the continuous *in vivo* monitoring of physiological analytes is described. The 2mm x 4mm x 0.5mm biochip contains two platinum working enzyme electrodes that adopt the microbore design to minimize diffusional limitations associated with enzyme kinetics. This configuration permits either dual analyte sensing or a differential response analytical methodology during amperometric detection of a single analyte. The working enzyme electrodes are complemented by a large area platinized platinum counter electrode and a silver reference electrode. The biorecognition layer of the working electrodes was fabricated from an ca. 1.0  $\mu\text{m}$  thick composite membrane of principally tetraethylene glycol (TEGDA) cross-linked poly(2-hydroxyethyl methacrylate) that also contained a derivatized polypyrrole component and a biomimetic methacrylate component with pendant phosphorylcholine groups. These two additional components were introduced to provide interference screening and *in vivo* biocompatibility respectively. This composite membrane was used to immobilize glucose oxidase and lactate oxidase onto both planar and microbore electrode designs, which were then used to assay for *in vitro* glucose and lactate, respectively. The glucose biosensor exhibited a dynamic linear range of 0.10 - 13.0 mM glucose with a response time ( $t_{95}$ ) of 50 s. The immobilized glucose oxidase within the hydrogel yielded a  $K_{m(\text{app})}$  of 35 mM, not significantly different from that for the native, solution-borne enzyme (33 mM). The microbore biosensor displayed linearity for assayed lactate up to 90 mM, which represented a 30-fold increase in linear dynamic lactate range compared to the biosensor with the planar electrode configuration. Preliminary *in vitro* operational stability tests performed with the microbore lactate biosensor demonstrated retention of 80% initial biosensor response after 5 days of continuous operation in buffer under physiologic conditions of pH and temperature.

INDEX TERMS: Implantable, subcutaneous, amperometric, biochip, microbore, hydrogel, glucose, lactate

**1. Introduction**

New concepts and developments in biosensor design and construction have emerged at a tremendous rate over the last two decades [1]. Motivated by a number of factors [2], the field of medical diagnostics has undoubtedly been the greatest beneficiary of these developments in biosensor technology [2]. The progress and development of clinically useful biosensors have been spear-headed by development of the glucose biosensor, which has dominated the commercial clinical market since the 1960s and was achieved through the early integration of the redox enzyme glucose oxidase with an oxygen electrode [3]. Since then, numerous materials, sensing configurations, and fabrication techniques have been reported for the construction of biosensors for glucose detection [4-10]. The reason for this high demand is the escalating mortality rate and life-threatening complications associated with the disease diabetes mellitus, which accounts for about 300 million sufferers worldwide [7]. This pandemic is characterized by failure to maintain blood glucose at suitable levels, primarily due to pancreatic dysfunction. There is hope that effective treatment to maintain normoglycemia and tighter blood glucose control will forestall some of the

long-term complications of the disease, including retinopathy, nephropathy and neuropathy. The evolution of glucose assay methods has been the central dogma for the development of present therapies for diabetes mellitus. At present, blood glucose control is based on blood glucose assays, which, with available methodology, still requires frequent blood sampling with some associated degree of inconvenience. Studies have even suggested that some diabetics may choose not to strive for close blood glucose control because of the obtrusiveness of present blood sampling and assay [8]. Even with the most assiduous use of the present glucose assay methodology however, it may still not be feasible to maintain close blood glucose control. This is largely because blood sampling is rather infrequent compared to the rate of blood glucose fluctuations. In short, blood sampling provides a discrete rather than a desired continuous blood glucose record, and is nowhere near sufficiently frequent and regular to serve as a warning of rapid blood glucose imbalances. Truly responsive therapies require continuous monitoring of glucose that may be achieved with implantable biosensor devices. Subcutaneously implanted biosensors measure interstitial glucose, which is arguably physiologically more relevant than blood glucose [11, 12]. This would enable therapeutic options that are not presently available.

To date, the development of a stable, highly accurate and continuous implantable biosensor for glucose that is acceptable to diabetic patients remains a significant challenge to researchers [11]. This is primarily due to device biocompatibility and signal interference-related issues. Thus far, the most promising results have been obtained with biosensors that are based on amperometric detection of nascent hydrogen peroxide produced by enzymes immobilized on electrodes or the alternative that is based on voltammetrically discharged oxygen at enzyme-modified electrode. To our knowledge, the glucose biosensor that has achieved the greatest longevity when subcutaneously implanted has been that designed by Updike *et al.* [13]. This device achieved a clinically useful lifetime of up to 5 months after implantation in dogs. The corresponding longest functionally lifetime attained with the oxygen-based sensor has been 108 days [14].

Following injury that results in tissue hypoxia, interstitial lactate levels increase and are the main source of metabolically-produced acid responsible for tissue acidosis. Lactate levels have also been found to correlate with the severity of injury, including hemorrhage [15, 16]. Small molecules such as lactate and glucose exist in equilibrium between the interstitial and vascular compartments when systemic levels are elevated. Interstitial levels of lactate should thus be reflective of systemic levels when whole body tissue hypoxia occurs such as in hemorrhage. The ability to monitor *in vivo* lactate in the interstitium is possible but current methods are either not clinically feasible for prolonged measurement or are unreliable because they suffer from deleterious effects of endogenous interferences and fouling.

Several lactate oxidase-based enzyme biosensors that detect lactate have been described in the literature [17-20]. However, these lactate sensors are prone to interfering reactions from coexisting electroactive species. A few enzymatic lactate probes are working in commercial analyzers [21-23]. Besides these, only a very few reliable possibilities of lactate analysis, without any pre-analytics and using a portable probe, are available for intermittent lactate concentration analysis. A rapid lactate measurement, which is ideally equivalent to real time monitoring, is of great importance; for the faster the lactate analysis, the better the diagnostic information. A lactate sensor for real-time monitoring should be specific for lactate, should need only one calibration before and/or after the invasive application and should be stable over a period of at least 5 hours in short-term application, more than 3 days for medium-term monitoring; e.g. during the postoperative period, and up to three months for long-term observation. Very few real-time, invasive, needle-type, lactate-sensing, catheter electrodes that demonstrate the aforementioned characteristics have been described in the literature. Hu *et al.* [24] were successful with a  $H_2O_2$  detecting needle for monitoring the subcutaneous lactate level. Baker and Gough [25] used oxygen detection. However, both versions of these lactate sensors are only suited for short-term continuous *in vivo* lactate monitoring. The ideal lactate sensor would be capable of both short-term as well as long-term continuous sensing, with the added clinical benefit of responding rapidly to an insult such as severe hemorrhagic shock.

The physiologic relationship between lactate and glucose is apparent in the Cori cycle [26]. Under conditions in which oxygen supply is limiting, e.g., in exercising muscle, or in the absence of mitochondria, e.g., in red blood cells, re-oxidation of NADH produced by glycolysis cannot be coupled to generation of ATP. Instead, its re-oxidation is coupled to the reduction of pyruvate to lactate. This lactate is released into the blood, and is taken up primarily by the liver, where it is oxidized to pyruvate and can be used for gluconeogenesis, wherein two molecules of pyruvate are used to synthesize one molecule of glucose. The



significance of these two metabolites and their intricate relationship as gauges for disease or chronic illness has been the subject of recent investigation and is still to be fully understood [27, 28].

In this paper, we report on the evaluation and analytical performance of three biosensor motifs; (i) a planar glucose biosensor fabricated from our "bio-smart" hydrogel formulation [29], (ii) a planar lactate biosensor containing lactate oxidase entrapped within cross-linked poly(2-hydroxyethyl methacrylate) and polyethylene glycol methacrylate, and (iii) a microbore lactate biosensor containing the hydrogel formulation used with the planar electrode design. We address the stability of lactate oxidase by enzyme molecular engineering ("PEGylation"), which enhanced solubility and stability in our membrane formulation. We also describe the design and fabrication of a combined glucose and lactate, dual-sensing, microfabricated biochip containing two microbore working electrodes, each with its respective counter electrode, and both with a common Ag/AgCl reference electrode. Each working electrode area was subsequently coated with a composite hydrogel formulation containing physically entrapped oxidase enzyme. The product of the enzymatic reaction was detected via constant potential amperometry. The microbore design for the working electrodes is expected to promote hemispherical diffusion over linear diffusion of substrate and/or product to the electrode surface with the consequence of an impact on dynamic range, sensitivity and response rate of the sensors. The composite polymer membrane of the biorecognition layer has been developed to incorporate the interference shielding capabilities conferred by electroactive polypyrrole [30] and the biocompatibility conferred by biomimetic phosphorylcholine derivatives [31-33] associated with the natural cell membrane. We demonstrate broader dynamic range and increased stability with our approaches. Our programmatic goal is to achieve stable, continuous, implantable long-term biosensors for the detection and quantitation of clinically important analytes such as glucose and lactate for periods of up to six weeks.

## *2. Biochip Design and Construction*

The die of the final implantable biochip is shown in Figure 1. Using techniques based on standard microfabrication technology, two miniature microbore enzyme electrodes, along with their respective platinum counter electrodes and a common Ag/AgCl reference electrode, were prepared on the single biochip substrate of insulating and chemically resistant borosilicate glass. The overall physical dimensions of the biochip substrate are 4.0 mm x 2.0 mm x 0.5 mm (L x W x T). The chip was prepared in a multi-stage photolithographic process employing two masks. The first metallization stage involved deposition of 100 Å of Ti/W (adhesion layer) followed by 1,000 Å of platinum metal via electron-gun sputtering. A positive photoresist was then spun applied and hard baked. The first of two optical photomasks was then aligned over the borosilicate glass substrate so as to produce the electron-gun sputtered platinum pattern into the photoresist and the resist developed to expose the underlying metal. The exposed metal was removed by ion-beam milling. Five bonding pads, each of dimension 300 µm square, were patterned 100 µm apart and 50 µm from the edges of the borosilicate glass die substrate. The working electrodes are arranged as a pair of disks of diameter 250 µm. This design permits either the simultaneous measurement of two different analytes or the opportunity to implement a differential response analytical methodology during amperometric detection of a specific analyte. The counter electrodes were precisely patterned to fully encapsulate their respective working electrode disk area. The diameter of each semicircular counter electrode was 1,150 µm, resulting in an effective area that is 125 times the active area of each working 'microbore' disk electrode. These dimensions satisfy the fundamental requirement that the counter electrode be able to pass sufficient current into the contained medium without requiring an excessive cell voltage or creating a non-uniform current distribution at the working electrode. A common silver electrode was similarly patterned on the biochip to form the third electrode of a three-electrode electrochemical cell. This completed the design of a co-planar configuration and considerable miniaturization of our previously demonstrated biosensor electrodes [29, 34-36]. A grid of platinum lines provided the electrical connection between electrodes and their respective bonding pads.

The borosilicate glass wafer containing the above electron-gun sputter-deposited and patterned metallic electrodes was then coated with a passivating layer of 0.5 µm silicon nitride layer. To define the microbore pattern to the circular metallic electrode area, to passivate the various conductive traces, and to reveal the other active metallic areas and bonding pads, the second of two optical photomasks and photopositive photoresist was employed to allow through-holes of diameter 10 µm to be fluoro-plasma etched into the silicon nitride layer above each working disk electrode. These bore holes were arranged in a

hexagonal-close packed (hcp) array, with the spacing between holes of 20  $\mu\text{m}$  from center on center (Figure 1). Such an arrangement yields the maximum microbore hole packing density of 74% within the defined working area. With the hcp arrangement, a total of 127 holes were accommodated within each working electrode area, resulting in an effective working electrode area of  $9.98 \times 10^{-3} \text{ mm}^2$ . The silicon nitride layer was also simultaneously etched away at the reference and counter electrode positions to expose the underlying sputtered metal and the bonding pads. The chip was finally completely chemically modified with an organosilane adhesion promoting layer of either methacryloxypropyl trimethoxysilane or  $\gamma$ -aminopropyl trimethoxysilane followed by coupling to acryloyl (polyethylene glycol) NHS ester. The former derivatizes the biochip surface for direct covalent coupling to the composite hydrogel sensing membranes, while the latter approach promotes hydrogen bonding to the composite gels.

### 3. Biochip Packaging

The biochips were packaged into suitable chip carriers, ultrasonically welded to suitable silicone-sheathed 24 gauge cables, and rendered biocompatible by surface modification using our polymer coating prior to implantation into animal models. The entire chip was threaded into the lumen at one end of a 0.5 mm diameter silicone tube having a 1  $\text{mm}^2$  cutout area to expose the sensing layers of the chip. The use of the novel methoxypropyl cyanoacrylate surgical adhesive TISSUMEND<sup>TM</sup> (VPL) for the attachment of the biochip to the inner lumen of silicone tubing and for the packaging seal should be suited to long-term implantation. This novel medical adhesive is specifically designed to counter the adverse effects of adhesion break-down over extended implantation time. Five copper lead wires that were previously bonded to the chip were encapsulated into a single ribbon cable that runs within the lumen of the silicone tube. The copper wires were exposed at the distal end of the tube via appropriate connectors for interfacing to an electrochemical workstation that allowed for dual or selective sensing, data collection and storage, of glucose and other clinical metabolites.

### 4. The Sensing Layer - Composite Hydrogel Membranes

Previous manuscripts [29, 36] detail the synthesis and characterization of composite hydrogel membranes composed of interpenetrating networks of inherently conductive polypyrrole grown within UV cross-linked hydroxyethyl methacrylate (HEMA)-based hydrogels. Entrapment of the appropriate oxidase enzyme within these composite materials and subsequent deposition onto platinum electrodes has produced clinically demonstrated amperometric biosensors for glucose, cholesterol, and galactose. These *in vitro* biosensors demonstrated excellent analytical characteristics of; expanded linear response range ( $10^{-5}$  –  $10^{-2}$  M), rapid response times (< 60 s), and good reproducibility and recovery. The polymer composite also provided a biocompatible microenvironment for the entrapped enzyme that afforded retained bioactivity (> 70 %) over a storage period of one year and was efficient in screening out common endogenous and exogenous physiologic interferents [30].

In the current design of the *in vivo* biochip the biorecognition layer uses a previously published polymer membrane formulation [29, 36] that was modified in three important ways. Firstly, the pyrrole component of the monomer formulation was supplemented by a unique bi-functional pyrrole monomer, 2-methacryloxyloxyethyl pyrrolyl butyrate. This bi-functional monomer was synthesized in our laboratories by the esterification of 1-H-pyrrole-3-butyric acid (also synthesized in our laboratories) with 2-hydroxyethyl methacrylate (commercially available) as shown in **Scheme 1**. This bi-functional monomer has a UV-polymerizable methacroyl terminus and an oxidatively polymerizable pyrrolyl terminus and may serve as a cross-linker between poly(HEMA) chains and polypyrrole chains. Polymerization of this derivative will covalently secure the electroactive polypyrrole component to the hydrogel network, thus preventing any possible *in vivo* leeching of the conductive polymer component from the composite membrane.

Secondly, to provide for long-term stabilization of membrane-immobilized oxidoreductases, a polyethylene glycol methacrylate (PEGMA) monomer was added to the bio-smart hydrogel formulation. This establishes PEG chains, set pendant to the polymer network, in the form of network supported polymer brushes. PEG chains are well known to stabilize proteins, preventing their denaturation and promoting long-term bioactivity [37-39].

The third alteration to the previously published bio-smart hydrogel membrane composition directly addresses the paramount issue of *in vivo* biocompatibility of the biochip devices. The body's physiologic response to a newly implanted sensor is essentially similar to that associated with wound healing [6]. Thus the "foreign" surface of the sensor provokes (amongst other things) the formation of blood clots within

seconds, minutes or hours leading to a progressive decline in sensor sensitivity that eventually culminates in sensor malfunction or "sensor demise". Of all the approaches that have been attempted to enhance biocompatibility, that of 'biomembrane mimicry or bio mimicry' appears to be, arguably, the most attractive solution for long-term implantable sensor surfaces [6, 40]. In biomimicry the chemical entities used are similar in molecular structure, form, and function to the lipids that comprise cell biomembranes and thus they present to the extracellular matrix the same chemical functionality and structure that emulates contact with the extracellular matrix [41]. The inclusion of a biomimetic constituent such as the phospholipid containing monomer, 2-methacryloxyethyl phosphorylcholine (MPC), into the composite hydrogel confers enhanced biocompatibility. While protein adsorption is expected to occur and indeed cannot be avoided in subcutaneous implantation, it is the denaturation of adsorbed proteins at the surface that initiates the inflammatory cascade. The goal is to accommodate protein adsorption while avoiding protein denaturation and aberrant conformational change. The protein conditioning layer provided by the MPC component mediates cellular response at the surface, which will be the major determining factor in the biocompatibility of the implanted surface, as viewed by the surrounding tissues. The use of these MPC polymers for biomimicry has been met with much success. Ishihara and coworkers [31-33] have demonstrated these biomedical polymers to suppress protein adsorption and platelet adhesion and activation and significantly improve the blood and tissue compatibility of *in vivo* biomedical devices coated with the MPC polymers.

The monomer cocktail comprising the various methacrylate components and the cross-linking agent, summarized in Table 1, served as the 'receiving' mixture for the oxidase enzyme and photoinitiator. The cocktail was precisely solvent cast over each sensing electrode area of the biochip with the aid of a computer-controlled robotic micro-array spotter (Cartesian Technologies, Inc., California). UV-induced polymerization of the methacrylate components followed immediately by electrochemical polymerization of the derivatized pyrrole functionality produced the final composite membrane structure shown in Figure 2.

#### 5. Fabrication of Glucose and Lactate Biosensors

Three biosensor systems were demonstrated; i) a planar glucose biosensor fabricated from the composite formulation containing the MPC and PEGMA components, ii) a planar lactate biosensor containing lactate oxidase entrapped within cross-linked p(HEMA) and PEGMA, and (iii) a microbore lactate biosensor containing the hydrogel formulation used with the planar electrode design in (ii). Table 2 summarizes the composition of each sensing membrane. Glucose oxidase (30 mg, Type VII-S) (E.C. 1.1.3.4. from *Aspergillus niger*, 150,000 U/mg solid) was dissolved in ethylene glycol:water (1:1 v/v) and then introduced into the receiving monomer cocktail comprising HEMA, PEGMA, TEGDA, MPC, and Py in a mole ratio 80:2.5:10:2.5:5 mol%. Photoinitiator dimethoxyphenyl acetophenone (4 wt%) was then completely dissolved in the mixture and 3  $\mu$ L of the enzyme-monomer mixture was applied to the surface of a planar platinized platinum electrode. UV irradiation (2.3 W/cm<sup>2</sup>, 366 nm, UVP Model CX-2000) was performed for 10 minutes to polymerize the methacrylates followed immediately by electropolymerization in 0.4 M pyrrole solution (PBKCl, pH 6.0, 0.1 M in each component) at +850 mV (vs. Ag/AgCl) to grow the polypyrrole component within the cross-linked methacrylate network.

With respect to the lactate biosensors, 1.4 mg lactate oxidase (E.C. 1.1.3.2 from *Pediococcus* species, 37 U/mg solid) was introduced into a HEPES buffer solution (5  $\mu$ L, 0.01 M, pH 7.4) containing 0.27 mg of an acryl-polyethyleneglycol(PEG)-NHS ester, MW 3400 (Nektar Therapeutics, Huntsville, AL). The enzyme and PEG components, in a mole ratio of 1:4, were allowed to couple at room temperature in the dark for 2.5 h through the amine and NHS-activated carboxylic functionalities, respectively. **Scheme 2** summarizes this enzyme PEGylation reaction. Subsequently, 5  $\mu$ L of a methacrylate mixture of HEMA, PEGMA, and TEGDA (96:1:3 mol%) containing dissolved photoinitiator was added to the above solution, and 3  $\mu$ L of the derivatized enzyme-monomer mixture applied to the active electrode areas of both a planar platinized platinum electrode (0.2 cm<sup>2</sup>) and a platinized platinum microbore electrode (ca. 0.04 cm<sup>2</sup>). Figure 3 shows both these electrode designs. The electrodes were then irradiated with UV light for 10 min to effect polymerization of the bioactive monomer mixture.

#### 6. Results and Discussion

Replicate testing of the amperometric response of the bio-smart composite hydrogel glucose biosensor in PBS 7.4 buffer solution to various glucose concentrations yielded a dynamic linear range of 0.10 - 13.0mM glucose with the following regression equation ( $n = 12$ ,  $r^2 = 0.994$ ):

$$\text{Biosensor response } (?A) = 0.5906 [\text{glucose}] (\text{mM}) + 0.1624 (\mu A)$$

The time taken to reach 95% of the steady state response was 50 s. This compares favorably to a  $t_{95}$  of 40 s for the previously published composite hydrogel biosensors employing only polypyrrole and cross-linked p(HEMA) [29]. To determine whether the sensor response was governed by mass transport of both substrate and product (diffusion controlled) or whether it was governed by the enzyme kinetics (kinetically controlled), the calibration data was represented in a Lineweaver-Burk plot (Figure 4). From the linearity of this plot over all glucose concentrations investigated, it can be concluded that Michaelis-Menten kinetics of the enzymatic reaction is the rate-limiting factor under these experimental conditions. The apparent Michaelis-Menten constant ( $K_{m(\text{app})}$ ) and maximum current ( $I_{\text{max}}$ ) estimated from the Lineweaver-Burk equation were 35 mM and 23.1  $\mu A$ , respectively. The  $K_m$  reported for free, solution-borne glucose oxidase is 33 mM [42]. This suggests that the composite hydrogel membrane does not impede or hinder the formation of enzyme-substrate complex, nor does it provide diffusional limitation to the glucose flux. For comparison, the  $K_{m(\text{app})}$  value for previously published composite hydrogel glucose biosensor was 43.7 mM with an  $I_{\text{max}}$  value of 19.42  $\mu A$  [29].

The pH and temperature dependent profiles in response to 10 mM glucose for the present glucose biosensor are shown in Figures 5 and 6 respectively, along with corresponding profiles for the solution-borne enzyme and the first generation composite hydrogel glucose sensor. The immobilized enzyme retains greater than 78% activity over the entire pH range investigated (4.5 – 7.5), with a pH optimum around 5.5 – 6.0, similar to that reported for solution-borne glucose oxidase [43]. With respect to the temperature profile, greater than 80% activity was retained over the range 20 – 50°C, with an optimum around 45°C. This represents about a 15°C shift towards a higher temperature performance when compared to free glucose oxidase [43]. Interestingly, with the inclusion of the MPC and PEGMA components, there is greater retention of enzyme activity at higher temperatures compared to previous membranes containing only polypyrrole and cross-linked p(HEMA) [29]. The inclusion of polyethylene glycol chains and zwitterionic phosphorylcholine moieties, both set pendant to the network backbone and at a level of 2.5 mol % respectively, appear to have little impact on the performance of hydrogel immobilized glucose oxidase. Studies are in progress to characterize the *in vivo* biocompatibility of these composite hydrogel membranes.

To determine the effect, if any, that the electrode geometry has on the analytical performance and characteristics of the enzyme biosensor, a planar platinized platinum electrode (0.20 cm<sup>2</sup>) and a platinized platinum microbore electrode (0.04 cm<sup>2</sup>) were both evaluated as substrates onto which were deposited a common lactate oxidase containing hydrogel. Figure 7 shows the corresponding response curves normalized to the active electrode area. The planar lactate biosensor demonstrated linearity to lactate concentrations prepared in phosphate buffered saline up to 3 mM with the following regression equation ( $r^2 = 0.980$ ):

$$\text{Biosensor response } (\mu A) = 0.0662 [\text{lactate}] (\text{mM}) + 0.021 (?A)$$

The sensor had a detection limit of  $8.0 \times 10^{-5}$  M ( $3S_{y/x}/\text{slope}$ ) and a response time ( $t_{95}$ ) of 20 s. In contrast, the microbore lactate biosensor exhibited an extensive linear dynamic response range up to 90 mM lactate with the following regression equation ( $r^2 = 0.994$ ):

$$\text{Biosensor response } (\mu A) = 0.0033 [\text{lactate}] (\text{mM}) - 0.001 (\mu A)$$

The detection limit of the microbore biosensor was determined to be  $8.0 \times 10^{-4}$  M ( $3S_{y/x}/\text{slope}$ ), with a response time ( $t_{95}$ ) of 50 s. Thus by changing the electrode design (and corresponding active electrode area) from a planar area to a microbore configuration, we incur a reduction of the flux of enzymatically generated H<sub>2</sub>O<sub>2</sub> to the underlying exposed platinized regions of the polarized platinum electrode. However, we simultaneously promote spherical/radial diffusion to the microbore array elements of the microbore electrode. This has the effect of extending the linear response range of the microbore biosensor by as much as 30 times that achieved by the planar biosensor. Figure 8 schematically represents the effect of electrode geometry on the mass-transport diffusional characteristics of the substrate at the active electrode area. The platinized sites of the microbore electrode (Figure 8a) provide microscopic surface topographies that promote radial mass transport of the substrate at the electrode surface. The platinized planar electrode geometry (Figure 8b) provides the equivalent electrode area that, in effect, causes the corresponding radial diffusion layers to overlap with one another and so produces the familiar, limiting situation of semi-infinite linear diffusion [44, 45].

When the potential of a planar electrode is stepped from a value at which no redox reactions occur to a value at which a diffusion-controlled oxidation occurs, the enzymatically generated  $\text{H}_2\text{O}_2$  molecules near the electrode surface are oxidized. The current decays as the electrolysis proceeds to deplete the solution near the electrode. This current can be described, under conditions of semi-infinite linear diffusion, by the Cottrell equation.

$$i = nFADC / \sqrt{\pi Dt}$$

The current is in direct proportion to the electrode surface area and  $i \rightarrow 0$  as  $t \rightarrow \infty$ .

With micron-scale diameter microelectrodes, the shape of the diffusion layer varies with the electrochemical technique in which the potential is applied to the electrode. With amperometry, as used in the present work, the electrode potential is fixed and diffusion of molecules can occur from regions directly adjacent to the electrode surface and outside the perimeter of the electrode. This latter type of diffusion, termed radial or spherical diffusion, predominates at long (seconds) time scales [46]. The steady-state current density,  $i_{ss}$ , associated with a recessed microbore or microdisk electrode is a function of the bulk concentration,  $C$ , of the electroactive species, the diameter,  $d$ , of the microbore or microdisk and the thickness,  $L$ , of the insulating coating [47]:

$$i_{ss} = \frac{8nFDC}{8L + \pi d}$$

The human body at rest produces on average 15-20 mmole/kg/day, with consumption occurring primarily in the liver and kidney. This creates a net normal blood lactate level of 0.5 – 2.0 mM. However, when cellular oxygen becomes limited, such as in hemorrhagic shock, pyruvate cannot be readily used in the Tri-Carboxylic Acid (TCA) Cycle and hence larger amounts undergo conversion to lactate via simple glycolysis [16, 48]. While the majority of electrochemical lactate biosensors that have been reported in the literature may have linear dynamic ranges that encompass the basal concentration range, far fewer have been reported with an upper limit approaching 100 mM [49] that has been achieved with the present microbore biosensor. The sensitivity of the microbore lactate biosensor is in the range typical of other existing amperometric lactate sensors [50-52]. This suggests that enzyme derivatization with PEG moieties and the composition of the sensing membrane do not compromise this analytical parameter of the biosensor. One direct consequence of enzyme PEGylation and subsequent “monomerization” is reflected in the stability profiles shown in Figure 9. Figure 9a shows storage stability data for underivatized vs. PEGylated LOx measured periodically over 3 months of storage at 4°C in the absence of buffer. These values are amperometric responses of both lactate biosensors to 10 mM lactate expressed as a % of the respective initial biosensor response. While both systems exhibit similar profiles within the first month of storage, the underivatized LOx system showed a much greater decline in activity beyond 30 days storage compared to the PEGylated LOx system. After 3 months of storage the PEGylated enzyme system retained 65% of its initial activity compared to only 50% for its underivatized counterpart. The operational stability of the microbore lactate biosensor was measured for 5 continuous days of operation in PBS at 37°C in response to a 10 mM lactate challenge. Figure 9b shows the corresponding biosensor response profile, with up to 80% retention of initial response after 5 days. Studies are currently in progress to evaluate the analytical performance of the microbore lactate biosensor implanted intramuscularly in a hemorrhagic rat model.

An additional major challenge, not addressed by the current design, is the sensor's performance dependence on tissue oxygen tension. To alleviate dependence of the biochip performance upon molecular oxygen tension when implanted *in vivo*, a redox mediator molecule, such as ferrocene monocarboxylic acid, may be covalently immobilized to the polymer hydrogel network. This moiety may be covalently immobilized as a pendant to the network backbone (through coupling to previously incorporated 2 methacryloyloxyethyl (polyethylene glycol) NHS ester) and with sufficient molecular flexibility and pendant chain length to allow ready diffusion to and from the FAD co-factor sites within the entrapped enzymes. Electrons from the redox center of the enzyme may then transfer to the redox sites of the polymer, with electron self-exchange between polymer redox sites allowing electron propagation to occur along a polymer chain segment or between chain segments to the electrode surface. Such a design is currently used in *in vitro* biosensor devices [53, 54].

## 7. Conclusion

We describe the design of a microfabricated biochip that integrates soft-condensed, bio-smart hydrogels with solid-state microfabricated electrodes that has the potential for the continuous, long-term, *in vivo* monitoring of clinically important analytes such as glucose and lactate. The device contains two microbore working electrodes that present the flexibility of dual analyte sensing or single analyte sensing using a differential response analytical methodology. The sensing membrane layer consists of, among other things, four main components; cross-linked p(HEMA) which serves as the hydrophilic network backbone, a polypyrrole network which acts as a barrier to interferents, PEG chains that are tethered to the polymer backbone to stabilize enzyme activity and an MPC component that confers improved tissue biocompatibility. By varying the nature of the oxidase enzyme, different amperometric biosensors may be conveniently fabricated and mass-produced. Our *in vivo* characterization aims at lactate and glucose. Preliminary *in vitro* data show that the composite formulation supports the development of a glucose biosensor with an immobilized enzyme layer that does not compromise enzyme kinetics. The microbore electrode design, when applied to a lactate biosensor, demonstrated linearity up to 90 mM lactate, thirty-fold greater than the linear dynamic range for a corresponding planar lactate biosensor. The effect of enzyme PEGylation was apparent in the relative ease of solubilization into the monomeric formulation prior to biorecognition membrane fabrication. Physical entrapment of the PEGylated enzyme into the bio-smart hydrogel to produce the lactate biosensor resulted in enhanced operational and storage stabilities of the PEGylated-enzyme biosensor compared to the underivatized enzyme biosensor. Based on these and previous *in vitro* studies, these biochips may exhibit the potential to function as stable, continuous, long-term, subcutaneously implanted biosensors for glucose, lactate, and other clinically important analytes. Such engineered devices promise to aid in the development of the much needed truly "closed-loop" insulin delivery system to replace pancreatic function in diabetics, as well as greatly enhance the many echelons of trauma care.

## 8. Acknowledgements

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# LIST OF TABLES

**TABLE 1:** The various monomers for the fabrication of bio-recognition composite membranes

MONOMER	RATIONALE	RANGE OF CONCENTRATION
Hydroxyethyl methacrylate (HEMA)	A highly hydrophilic monomer, widely used in biomedical applications such as contact lens fabrication	69 – 90 mol%
PEG monomethacrylate (PEGMA)	A hydrophilic monomer that stabilizes protein (enzyme) activity, reduces denaturation and promotes interfacial bio compatibility	5 – 30 mol%
Tetraethyleneglycol diacrylate (TEGDA)	A bi-functional monomer that is UV polymerizeable at both ends. This monomer strongly influences the cross-link density of the network.	1 – 12 mol%
2-methacryloxyloxyethyl phosphorylcholine(MPC)	A zwitterionic monomer that segregates to the interface and confers interfacial biocompatibility.	10 – 30 mol%
2-methacryloxyloxyethyl pyrrolyl butyrate (MPyBA)	A bi-functional monomer that is UV polymerizable on one end and oxidative polymerizable on the other. This influences the cross-link density of the network.	5 mol%
Pyrrole (Py)	Oxidatively polymerizeable monomer which when polymerized links with the bifunctional (MPy) to creates an interpenetrating network and confers interference shielding properties to the membrane.	5 mol%
Ferrocene monocarboxylic methacrylate	A bi-functional redox active mediator that is UV polymerizable and which will alleviate oxygen dependence <i>in vivo</i>	5 mol%

**TABLE 2:** The various monomers used for the fabrication of glucose and lactate biosensors

Compound	mol%		Mass (g)		Volume (mL)	
	glucose	lactate	glucose	lactate	glucose	lactate
HEMA	80.0	96.0	-----	-----	0.0332	0.0440
PEGMA	2.5	1.0	-----	-----	0.0575	0.0200
TEGDA	10.0	3.0	-----	-----	0.0068	0.0030
MPC	2.5	-----	0.0020	-----	-----	-----
Py	5.0	-----	-----	-----	0.0018	-----

## FIGURE CAPTIONS

**Figure 1.** Schematic illustration of the final microfabricated amperometric biochip device showing the borosilicate glass substrate after electron-gun sputter-deposition of platinum metal and the microbore design of the working electrodes showing the hexagonal-close packed arrangement of microbore array elements.

**Scheme 1.** Formation and structure of the bifunctional pyrrole monomer containing the methacrylate functionality for UV co-polymerization with hydroxyethyl methacrylate and pyrrolyl moiety for co-electropolymerization with pyrrole.

**Figure 2.** Partial structure of the final cross-linked hydrogel membrane comprising polyHEMA, polypyrrole, pendant MPC moieties, pendant PEG chains, and containing physically entrapped oxidase enzyme.

**Scheme 2.** Derivatization of oxidoreductase enzyme with activated PEG acrylate ester. LOx = lactate oxidase.

**Figure 3.** Photograph of the two different electrode designs: A) planar platinized platinum electrode, and B) platinized platinum microbore electrode.

**Figure 4.** Lineweaver-Burk plot of  $1/[\text{glucose}]$  ( $\text{mM}^{-1}$ ) versus  $1/I$  ( $\text{A}^{-1}$ ) performed in 0.1M phosphate buffered saline.

**Figure 5.** The pH profile of the present glucose biosensor (?) in response to 10 mM glucose. Superimposed on the plot are corresponding profiles for p(HEMA)/PPy/GOx (?) (the previously published glucose biosensor (29)) and soluble GOx (■).

**Figure 6.** The temperature profile of the present glucose biosensor (?) in response to 10 mM glucose. Superimposed on the plot are corresponding profiles for p(HEMA)/PPy/GOx (?) (the previously published glucose biosensor (29)) and soluble GOx (■).

**Figure 7.** Comparison of response curves of lactate biosensors having a planar platinized platinum electrode, PME (■) and a platinized platinum microbore electrode design, MBE (♦) (normalized to active unit electrode area). Conditions were: 0.01M  $\text{KH}_2\text{PO}_4$  containing 0.01M KCl, pH 7.2, 28°C.

**Figure 8.** Schematic representation of the conversion of radial to linear diffusion of substrate at (A) the platinized microbore electrode geometry and (B) the platinized planar electrode geometry.

**Figure 9.** (A) Storage stability profiles for PEGylated lactate oxidase (■) and underivatized lactate oxidase (♦). Both enzymes were immobilized in cross-linked p(HEMA) hydrogel membranes and stored dry at 4°C when not in use. The amperometric responses of both systems were in response to a 10 mM lactate challenge in PBS, pH 7.4 at 37°C. (B) Operational stability profile for PEGylated lactate oxidase in response to 10 mM lactate challenge in PBS, pH 7.4 at 37°C.

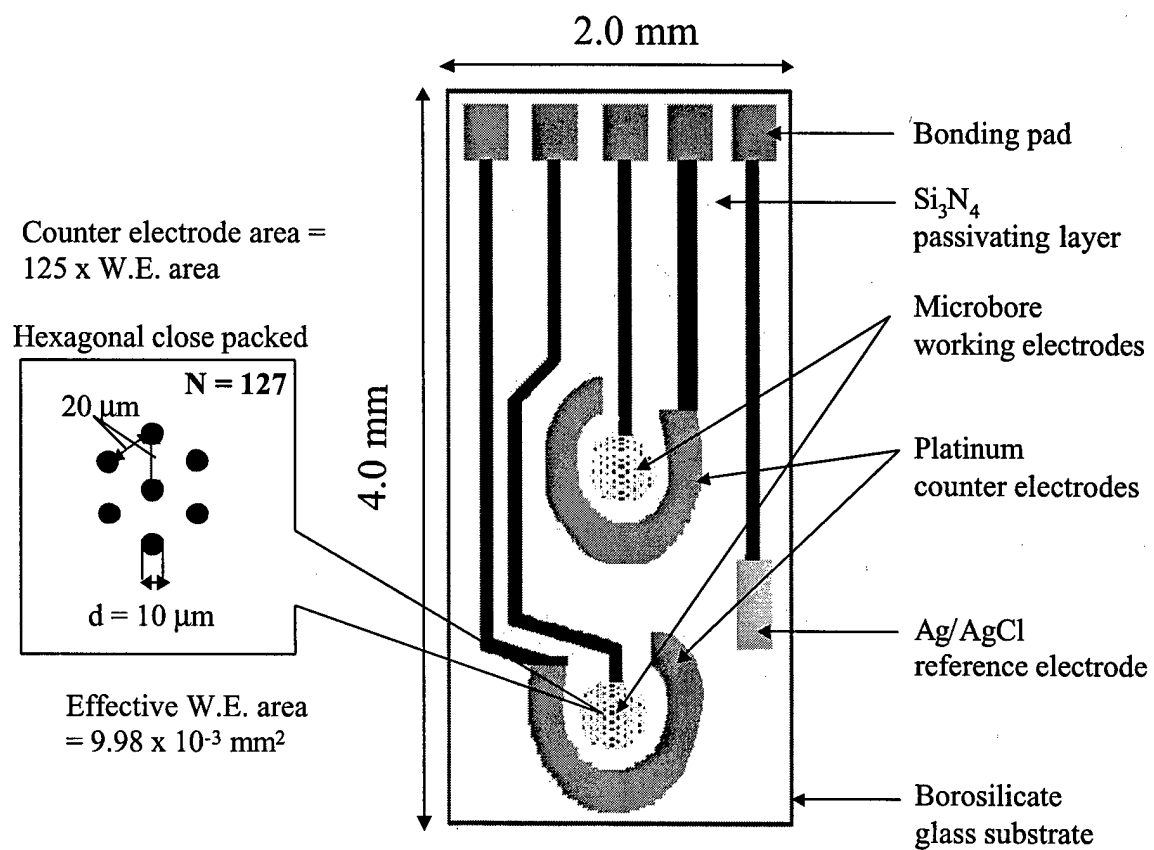
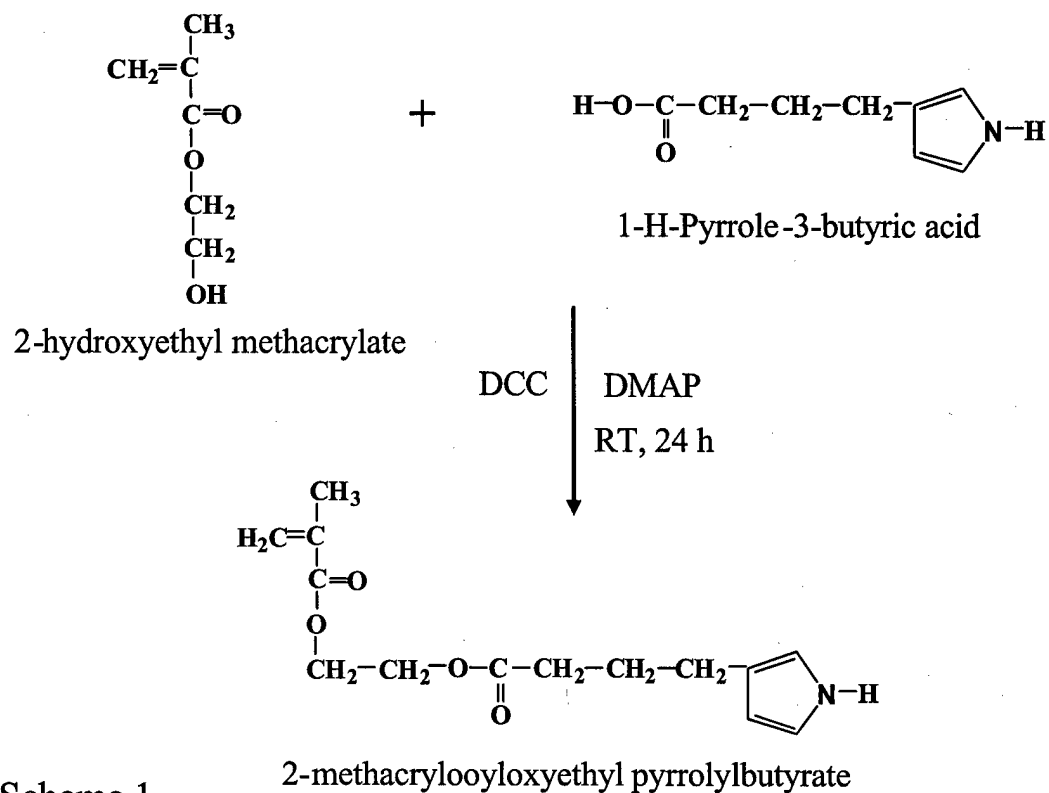


Figure 1



Scheme 1.

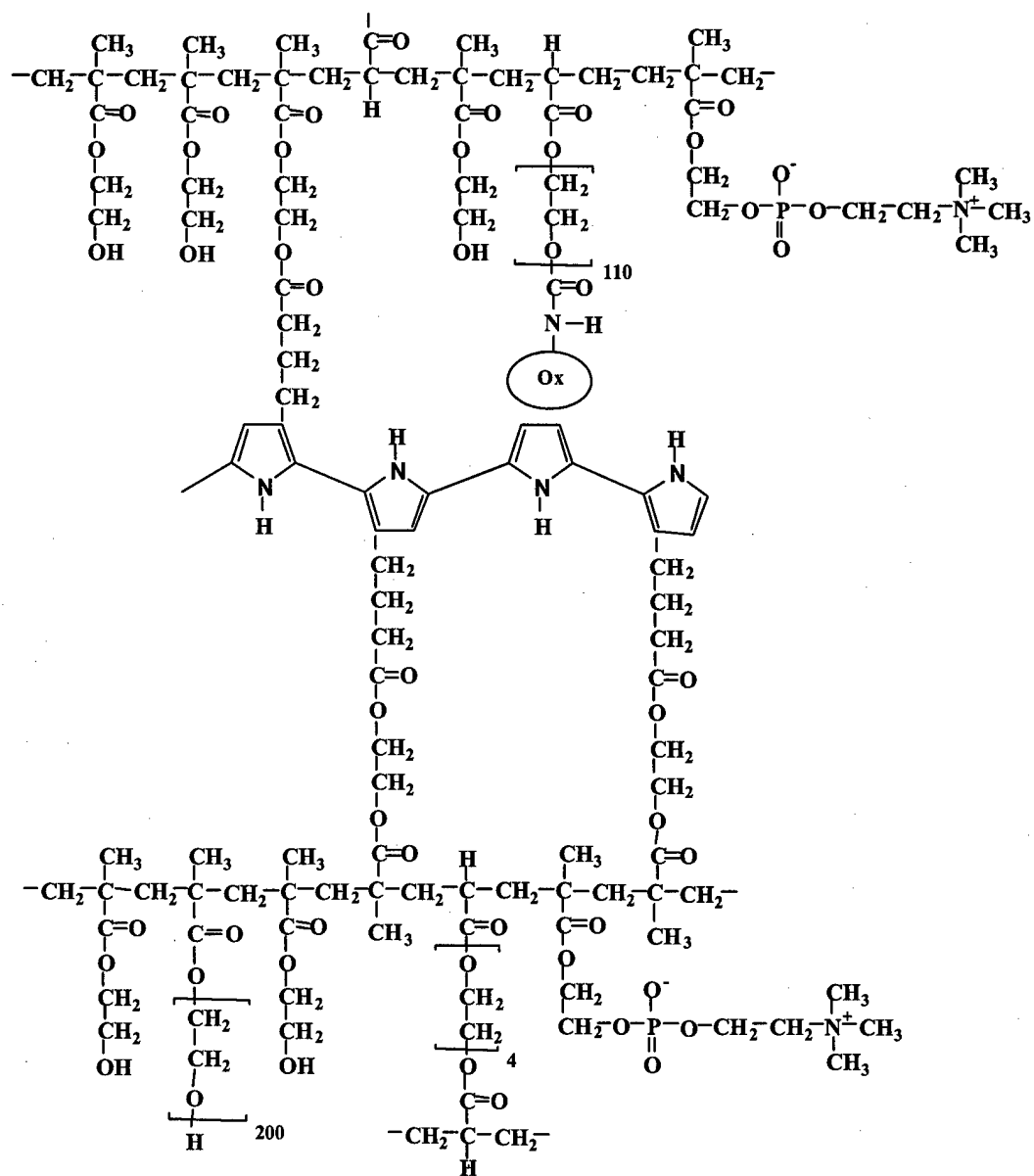
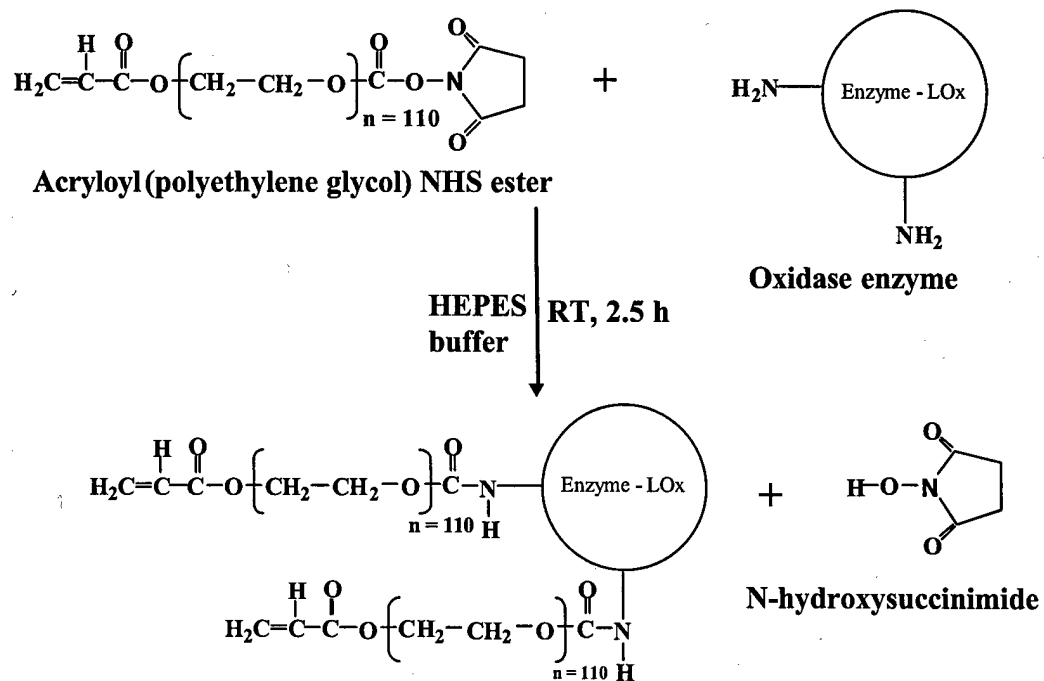


Figure 2



Scheme 2

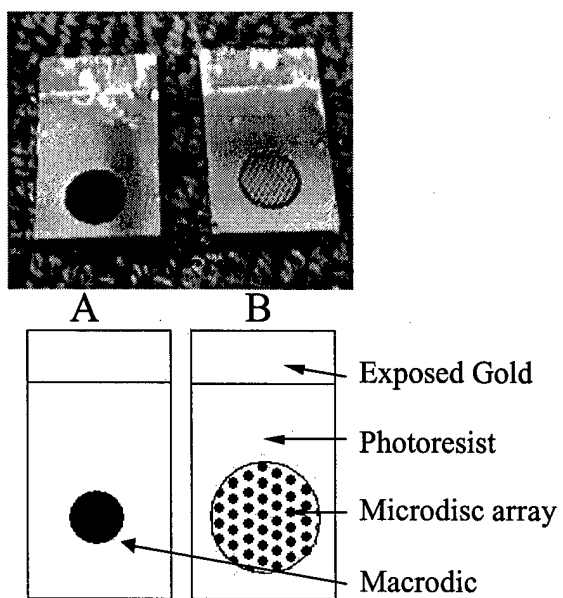


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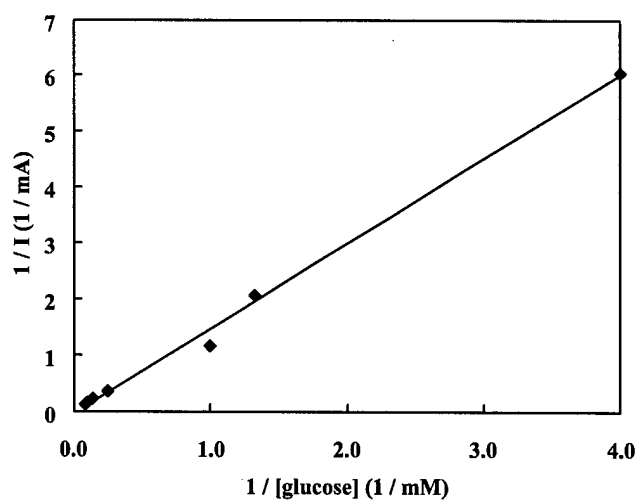


Figure 4



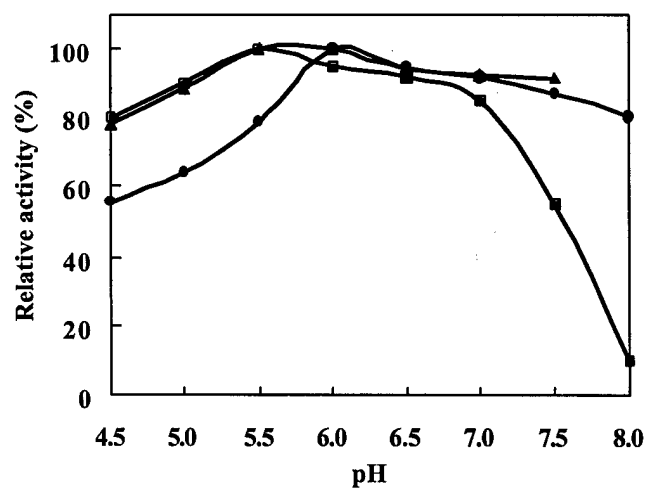


Figure 5

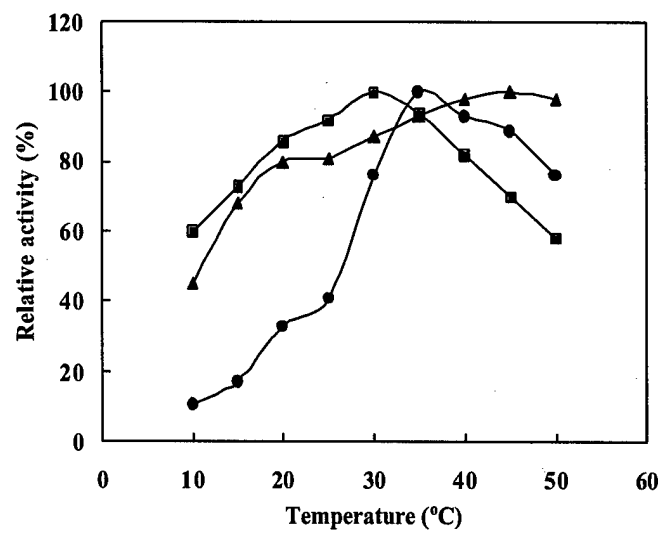


Figure 6

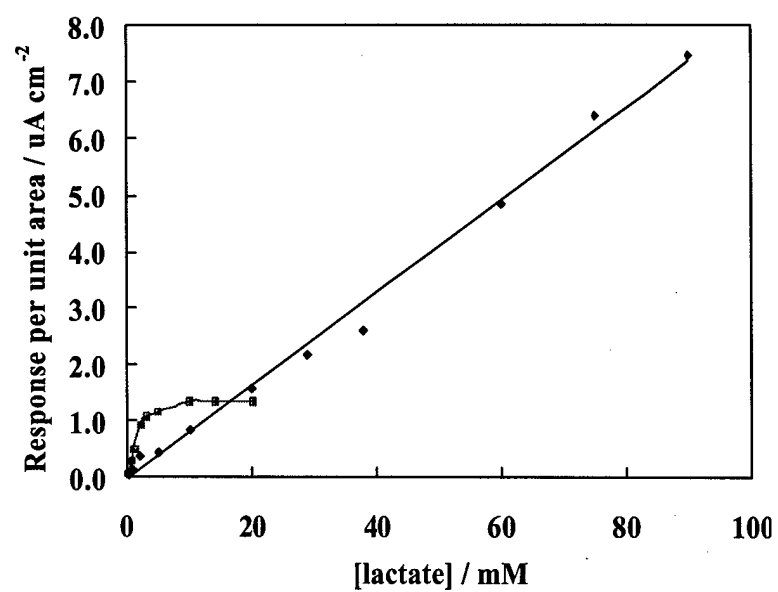


Figure 7

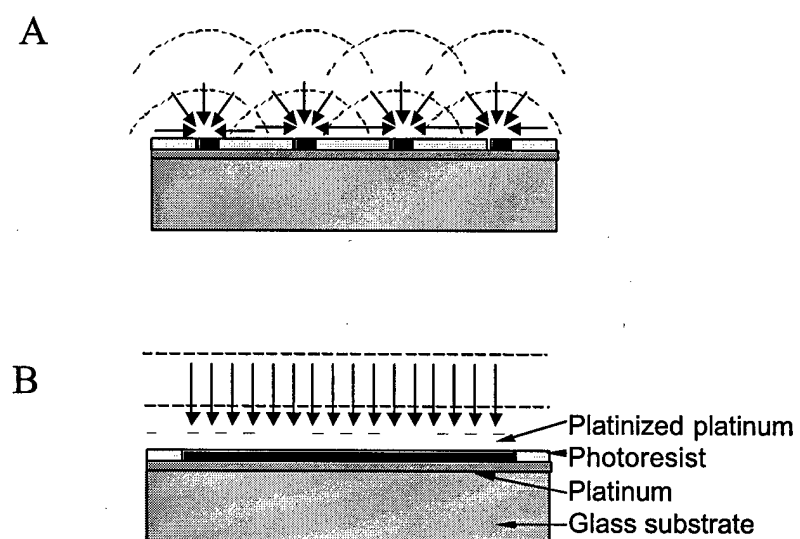


Figure 8

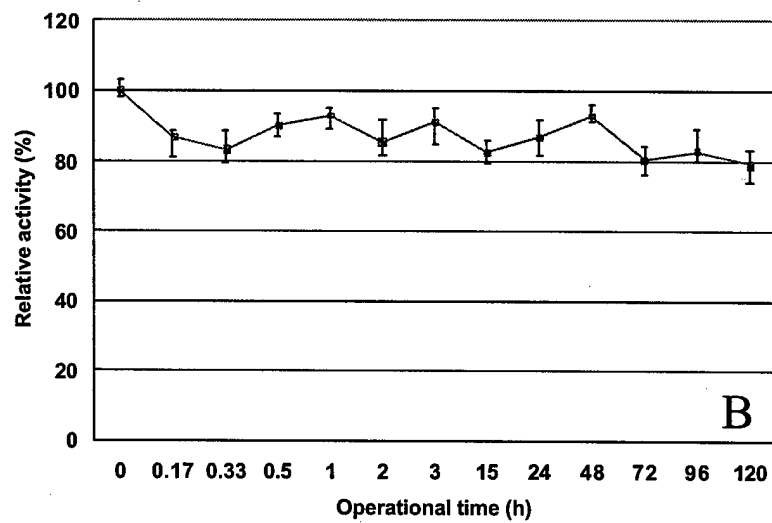
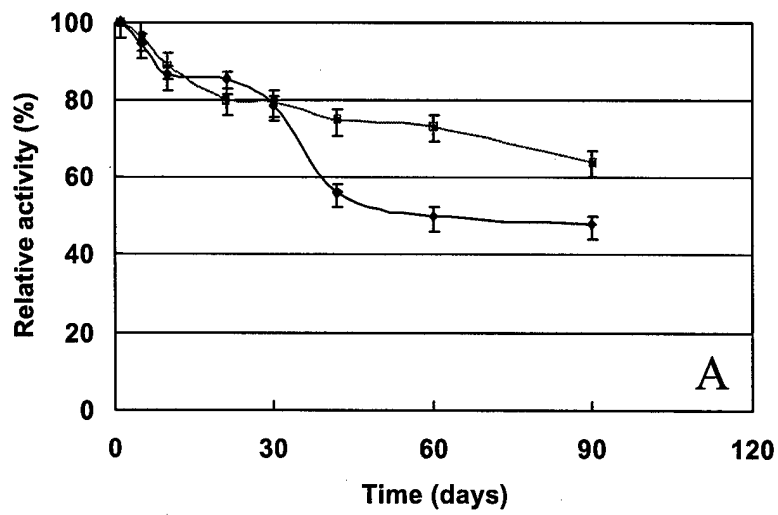
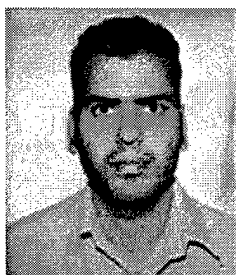


Figure 9



**Anthony Guiseppi-Elie** is Professor of Chemical Engineering and Professor of Emergency Medicine, Affiliate Professor of Biomedical Engineering and Director of the Center for Bioelectronics, Biosensors and Biochips at Virginia Commonwealth University. He is also President and Scientific Director of ABTECH Scientific, Inc., a biomedical diagnostics company located in Biotechnology Research Park, Richmond, Virginia. He holds the Doctor of Science degree in materials science and engineering from MIT, the Master of Science degree in chemical engineering from the University of Manchester Institute of Science and Technology (UMIST) and the Bachelor of Science degree (*First Class Honors*) with majors in Analytical Chemistry and Applied Chemistry from the University of the West Indies (UWI). Tony spent 15 years in intrapreneurial and entrepreneurial industrial research and development before becoming a professor at VCU in 1998. His research interests are in **engineered biosystems in the service of health and medicine**. This includes bioelectrochemistry and bioelectronic devices, bioactive hydrogels, biosensors and biochips for biomedical diagnostics, and high-throughput DNA/RNA screening and analysis. He has published ca. 70 technical papers, five book chapters and holds 8 patents. At VCU Prof. Guiseppi teaches materials chemistry, bioengineering, nanostructured materials and biotechnology. He is a member of the editorial boards of the *Journal of Bioactive and Compatible Polymers* and of *Your World*.

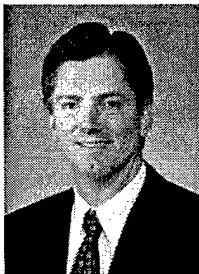


**Sean I. Brahim** is a Research Associate and Director of the Biosensors Laboratory in the Center for Bioelectronics, Biosensors and Biochips (C3B) at Virginia Commonwealth University (VCU). He received the B.S. degree (*First Class Honors*) in Chemistry (1995) and Ph.D. degree in Bioanalytical Chemistry (2001) from the University of the West Indies (UWI), St. Augustine, Republic of Trinidad and Tobago. He has over 9 years experience in the synthesis and *in vitro* characterization of biomaterials for biosensor applications. His research interests are in molecular engineering of smart materials for the development and application of materials-based chemical sensors, biosensors, biochips, and controlled release devices, and in the synthesis and chemistry of polymers at surfaces and interfaces as it relates to bio-immobilization and biocompatibility. He has published 17 papers.



**Ms. Gymama Slaughter** is a Graduate Research Assistant and C3B Scholar within the Center for Bioelectronics, Biosensors and Biochips and a Ph.D. Candidate in the School of Engineering at Virginia Commonwealth University. She holds the Bachelor of Science (*cum laude*) in Chemistry and Education (2001) and the Master of Science in Engineering (2003), both from Virginia

Commonwealth University. Her doctoral thesis research focuses on the development of cell-based biosensors using bio-impedance principles and signal processing applied to neurotoxicity sensing. She is a member of the Virginia Science Academy and the Golden Key National Honor Society.



**Kevin R. Ward** received the B.S. degree (*cum laude*) in Physiology from Louisiana State University in 1985 and the M.D. degree in Medicine from Tulane University School of Medicine, in 1989. He completed his Residency in Emergency Medicine from University of Pittsburgh in 1992 followed by a two-year resuscitation research fellowship at the Ohio State University in Columbus, OH. Before coming to VCU, Dr.

Ward was a senior staff member of the Henry Ford Health System in Detroit, MI. Dr. Ward's research interests include noninvasive biosensing and oxygen transport in shock to prevent the development of sepsis and multisystem organ failure. He presently serves as Director of Research in the VCU Department of Emergency Medicine and Associate Director of the VCU Reanimation Engineering Shock Center (VCURES).

**APPENDIX III – Poster that was presented at the ATACCC 2003 Meeting, Florida**

### Feasibility Studies in Development of a Temporary Implantable Lactate Biochip Sensor for Monitoring During Hemorrhage

Anthony Gargiulo, Ph.D.,<sup>1</sup> Scott Bortner, Steven Johnson, Gregory Strohman, Felix Mierisch, Kathy Flannigan, Rainer Simon, Robert Dugan, Leticia Fuent, R. Wayne Barker, Kevin Wilson,  
Department of Chemical Engineering and <sup>2</sup>Research Center for Bioelectronics, Biochemists and Biophysicists, Virginia Commonwealth University,  
Richmond, Virginia 23298-3038, <sup>3</sup>Microwave and Optical Systems Branch, NASA Glenn Research Center, Cleveland, Ohio 44135, <sup>4</sup>Electron Beam Technology Branch, NASA Glenn  
Research Center, Cleveland, Ohio, 44135, <sup>5</sup>Department of Biomedical, Virginia Commonwealth University, Richmond, Virginia, 23298, <sup>6</sup>Department of Emergency Medicine, Virginia  
Commonwealth University Resurrection Engineering Shock Center, Richmond, Virginia 23298

2008年12月15日

1. Different ways that people in some regions experience human rights are caused by economic, environmental, and social or economic factors. Liberty is the area where it would first be observed to understand the situation better.

2. Liberty is the area that leads toward the growth with the concept of human, individual, and economic rights in different ways on different social groups. The individual, human, and economic and social consequences of human rights are the way that people in different regions experience the situation. Liberty is the area where people experience the situation.

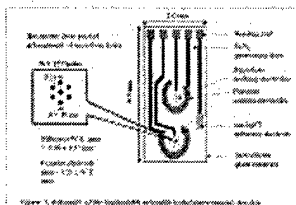
3. The area is the area that leads toward the growth with the concept of human, individual, and economic rights in different ways on different social groups. The individual, human, and economic and social consequences of human rights are the way that people in different regions experience the situation. Liberty is the area where people experience the situation.

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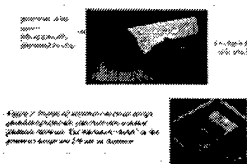
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Die folgende Tabelle enthält die Werte für die verschiedenen Variablen, die in der Tabelle angegeben sind. Die Werte sind in der Tabelle angegeben und sind in der Tabelle angegeben.



†*Figure 1* is available in the online edition of this article.

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### Abstracts of Speeches



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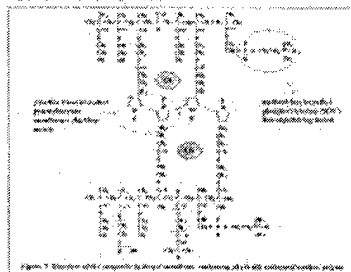
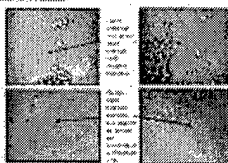


Figure 3. The effect of the concentration of the solution on the adsorption of the dye.

22. 2008年12月1日，甲公司以公允价值为1000万元的固定资产换入乙公司公允价值为800万元的固定资产，另收到乙公司支付的补价200万元。假定不考虑相关税费，甲公司换入乙公司固定资产的入账价值为（ ）万元。

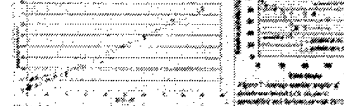
- to describe the transmission of the pathogen to another host, either by direct contact or by indirect contact (e.g. via fomites)
- the majority of the transmission takes in respiratory particles (droplets) or via contact with contaminated surfaces
- only some zoonotic agents are able to cause disease in both the animal reservoir and the human host




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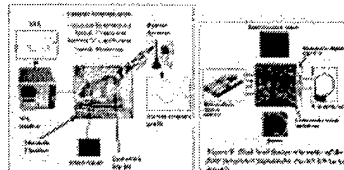


*Sedimental facies*



1. The proposed system is not feasible because it is too expensive and too complex.

|                                                                                                                                                                                                                                        |                                                                                                                                           |                                                                                                                                           |                                                                                                                                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| <p>  </p> <p> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ БІЛІМ ЖӘНЕ ҒЫЛЫМ МИНИСТРЛІГІ</b><br/> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ ҒЫЛЫМ ЖӘНЕ БІЛІМ МИНИСТРЛІГІ</b> </p> | <p> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ БІЛІМ ЖӘНЕ ҒЫЛЫМ МИНИСТРЛІГІ</b><br/> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ ҒЫЛЫМ ЖӘНЕ БІЛІМ МИНИСТРЛІГІ</b> </p> | <p> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ БІЛІМ ЖӘНЕ ҒЫЛЫМ МИНИСТРЛІГІ</b><br/> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ ҒЫЛЫМ ЖӘНЕ БІЛІМ МИНИСТРЛІГІ</b> </p> | <p> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ БІЛІМ ЖӘНЕ ҒЫЛЫМ МИНИСТРЛІГІ</b><br/> <b>ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ ҒЫЛЫМ ЖӘНЕ БІЛІМ МИНИСТРЛІГІ</b> </p> |
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